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(54) TGF-BETA FORMULATION FOR INDUCING BONE GROWTH

TGF-BETA ZUSAMMENSETZUNG ZUM HERBEIFÜHREN VON KNOCHENWACHSTUM

FORMULATION DU FACTEUR DE CROISSANCE DE TRANSFORMATION BETA PROVOQUANT LA CROISSANCE DES OS

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- **KEYSTONE SYMPOSIUM ON PROGRESS IN BASIC RESEARCH OF WOUND REPAIR AND ITS APPLICATION TO CLINICAL MANAGEMENT OF PROBLEMATIC WOUNDS. J. CELL BIOCHEM. SUPPL 0 (17 PART E). MARCH 29-APRIL 4 1993 , BRECKENRIDGE,COLORADO,USA. page 25 BECK LS ET AL. 'TRANSFORMING GROWTH FACTOR BETA-1 BOUND TO TRICALCIUM PHOSPHATE ACCELERATES BONE FORMATION WITHIN SKULL DEFECTS.'**
- **KEYSTONE SYMPOSIUM ON PROGRESS IN BASIC RESEARCH OF WOUND REPAIR AND ITS APPLICATION TO CLINICAL MANAGEMENT OF PROBLEMATIC WOUNDS, J. CELL BIOCHEM. SUPPL 0 (17 PART E), MARCH 29-APRIL 4, 1993, BRECKENRIDGE, COLORADO, US, page 25; BECK LS ET AL.: 'TRANSFORMING GROWTH FACTOR BETA-1 BOUND TO TRICALCIUM PHOSPHATE ACCELERATES BONE FORMATION WITHIN SKULL DEFECTS.'**

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Description

Background of the Invention

Field of the Invention

This invention relates to the use of transforming growth factor-beta (TGF-β) to induce bone growth *in vivo* and to formulations of TGF-β and tricalcium phosphate useful for this purpose.

Description of Related Art

The disorders associated with bone loss present major public health problems for Western societies. Osteoporosis alone may affect 20 million Americans in the early years of the next century. Hence, there is wide interest in identifying factors or potential therapeutic agents that inhibit bone loss and stimulate the formation of healthy new bone.

Bone is an extremely complex, but highly organized, connective tissue that is continuously remodeled during the life of an adult by cellular events that initially break it down (osteoclastic resorption) and then rebuild it (osteoblastic formation). This remodeling process occurs in discrete packets throughout the skeleton, i.e., in both cortical bone and trabecular bone. It has recently been reported that mouse bone marrow cells can be stimulated to generate osteoclasts in the presence of parathyroid hormone-related protein or vitamin D. See Akatsu et al., Endocrinology, 125: 20-27 (1989); Takahashi et al., Endocrinology, 123: 2600-2602 (1988) and Takahashi et al., Endocrinology, 123: 1504-1510 (1988).

The currently available therapeutic agents known to stimulate bone formation are fluoride, estrogen, and vitamin D. Fluoride clearly increases trabecular bone mass, but questions remain about the quality of the new bone formed, the side effects observed in some patients, whether there are beneficial effects on vertebral fracture rates, and whether increased fragility of cortical bone with subsequent propensity to hip fracture follows.

Another approach is using agents that promote resorption (parathyroid hormone) and then interrupt resorption (calcitonin). One proposed, but not validated, such sequential therapeutic regimen is coherence therapy, where bone metabolic units are activated by oral phosphate administration and then resorption is inhibited by either diphosphonates or calcitonin.

Within the past few years several factors that stimulate osteoblasts were identified in bone, including TGF-β, fibroblast growth factor, platelet-derived growth factor, insulin-like growth factor I, and β2 macroglobulin. Of these, TGF-β and IGF-I were deemed attractive candidates for factors linking previous bone resorption with subsequent bone formation. Mundy, The Journal of NIH Research, 1: 65-68 (1989).

Other proteins stored in the bone matrix may also be important for bone formation. When demineralized bone was injected into the muscle or subcutaneous tissue of rats, a cascade of events, including chondrogenesis, ensued. Urist, Science, 150: 893 (1965). This observed activity was due to bone morphogenetic protein (BMP). Since the 1960s several investigators have attempted to identify and characterize this activity. Thus, a protein of 22 Kd, called osteogenin, was identified that possessed the activity. Sampath et al., Proc. Natl. Acad. Sci. USA, 84: 7109 (1987). Three proteins from demineralized ovine bone matrix were identified as having this activity. Wang et al., Proc. Natl. Acad. Sci., 85: 9484 (1988) and Wozney et al., Science, 242: 1528 (1988). These proteins were named BMP-1, BMP-2A, and BMP-3, the latter two of which belong to the extended TGF-β family by limited sequence homology. These workers modified the assay for bone induction to show cartilage formation but did not show that the proteins ultimately stimulate formation of bone.

The TGF-β group of molecules are each dimers containing two identical polypeptide chains linked by disulfide bonds. The molecular mass of these dimers is about 25 Kd. Biologically active TGF-β has been defined as a molecule capable of inducing anchorage independent growth of target cell lines or rat fibroblasts in in vitro cell culture, when added together with EGF or TGF-α as a co-factor. TGF-β is secreted by virtually all cell types in an inactive form. This latent form can be activated by proteolytic cleavage of mature TGF-β from its precursor (at the Arg-Ala bond in position 278). A non-covalent complex is formed from the association of the mature TGF-β with the precursor remainder or with a protein binding to TGF-β or with alpha$_2$-macroglobulin. This complex is disrupted so as to activate the TGF-β either by exposure to transient acidification or by the action of exogenous proteases such as plasmin or plasminogen activator.

There are at least five forms of TGF-β currently identified, TGF-β1, TGF-β2, TGF-β3, TGF-β4, and TGF-β5. Suitable methods are known for purifying this family of TGF-βs from various species such as human, mouse, monkey, pig, bovine, chick, and frog, and from various body sources such as bone, platelets, or placenta, for producing it in recombinant cell culture, and for determining its activity. See, for example, Derynck et al., Nature, 316: 701-705 (1985); European Pat. Pub. Nos. 200,341 published December 10, 1986, 169,016 published January 22, 1986, 268,561 published May 25, 1988, and 267,463 published May 18, 1988; U.S. Pat. No. 4,774,322; Seyedin et al, J. Biol. Chem., 262: 1946-1949 (1987); Cheifetz et al, Cell, 48: 409-415 (1987); Jakowlew et al., Molecular Endocrin., 2: 747-755 (1988); Dijke et

al., Proc. Natl. Acad. Sci. (U.S.A.), 85: 4715-4719 (1988); Derynck et al., J. Biol. Chem., 261: 4377-4379 (1986); Sharples et al., DNA, 6: 239-244 (1987); Derynck et al., Nucl. Acids. Res., 15: 3188-3189 (1987); Derynck et al., Nucl. Acids. Res., 15: 3187 (1987); Derynck et al., EMBO J., 7: 3737-3743 (1988)); Seyedin et al., J. Biol. Chem., 261: 5693-5695 (1986); Madisen et al., DNA, 7: 1-8 (1988); and Hanks et al., Proc. Natl. Acad. Sci. (U.S.A.), 85: 79-82 (1988).

TGF-β3, TGF-β4, and TGF-β5, which are the most recently discovered forms of TGF-β, were identified by screening cDNA libraries. None of these three putative proteins has been isolated from natural sources, although Northern blots demonstrate expression of the corresponding mRNAs. TGF-β4 and TGF-β5 were cloned from a chicken chondrocyte cDNA library (Jakowlew et al., Molec. Endocrinol., 2: 1186-1195 [1988]) and from a frog oocyte cDNA library, respectively. The frog oocyte cDNA library can be screened using a probe derived from one or more sequences of another type of TGF-β. TGF-β4 mRNA is detectable in chick embryo chondrocytes, but is far less abundant than TGF-β3 mRNA in developing embryos or in chick embryo fibroblasts. TGF-β5 mRNA is expressed in frog embryos beyond the neurula state and in *Xenopus* tadpole (XTC) cells.

TGF-β has been shown to have numerous regulatory actions on a wide variety of both normal and neoplastic cells. TGF-β is multifunctional, as it can either stimulate or inhibit cell proliferation, differentiation, and other critical processes in cell function (M. Sporn, Science, 233:532 [1986]). For a general review of TGF-β and its actions, see Sporn et al., J. Cell Biol., 105: 1039-1045 (1987), Sporn and Roberts, Nature, 332: 217-219 (1988), and Sporn and Roberts, in Sporn and Roberts, ed., Handbook of Experimental Pharmacology: Peptide Growth Factors and Their Receptors I, Springer-Verlag, New York, pp. 3-15 (1990).

The multifunctional activity of TGF-β is modulated by the influence of other growth factors present together with the TGF-β. TGF-β can function as either an inhibitor or an enhancer of anchorage-independent growth, depending on the particular set of growth factors, e.g., EGF or TGF-α, operant in the cell together with TGF-β (Roberts et al., Proc. Natl. Acad. Sci. U.S.A., 82:119 [1985]). TGF-β also can act in concert with EGF to cause proliferation and piling up of normal (but not rheumatoid) synovial cells (Brinkerhoff et al., Arthritis and Rheumatism, 26:1370 [1983]).

Although TGF-β has been purified from several tissues and cell types, as indicated above, it is especially abundant in bones (Hauschka et al., J. Biol. Chem., 261: 12665 [1986]) and platelets (Assoian et al., J. Biol. Chem., 258: 7155 [1983]). TGF-β is postulated to be one of the local mediators of bone generation and resorption, because of its presence in large amounts in bone and cartilage, because cells with osteoblast and chondrocyte lineage increase replication after exposure to TGF-β, and because TGF-β regulates differentiation of skeletal precursor cells. See Centrella et al., Fed. Proc J., 2: 3066-3073 (1988). Immunohistochemical studies have shown that TGF-β is involved in the formation of the axial skeleton of the mouse embryo. TGF-β is also present in other embryos in the cytoplasm of osteoblasts in centers of endochondral ossification and in areas of intramembranous ossification of flat bones, such as the calvarium. Heine et al., J. Cell. Biol., 105: 2861-2876 (1987). Following in situ hybridization of TGF-β1 probes, localization of TGF-β in both osteoclasts and osteoblasts has been described in development of human long bones and calvarial bones. Sandberg et al., Development, 102: 461-470 (1988); Sandberg et al., Devel. Biol., 130: 324-334 (1988). TGF-β is found in adult bone matrix (Seyedin et al., Proc. Natl. Acad. Sci. USA, 82: 2267-2271 [1985], Seyedin et al., J. Biol. Chem., 261: 5693-5695 [1986]) and appears at the time of endochondral ossification in an *in vivo* model of bone formation (Carrington et al., J. Cell. Biol., 107: 1969-1975 [1988]). Cultured fetal bovine bone osteoblasts as well as rat osteosarcoma cells have high mRNA levels for TGF-β and secrete relatively high concentrations of TGF-β (Robey et al., J. Cell. Biol., 105: 457-463 [1987]).

In certain *in vitro* models, TGF-β was found to stimulate the synthesis of collagen, osteopontin, osteonectin, and alkaline phosphatase, and to stimulate replication in osteoblast-like cells. See Centrella et al., J. Biol. Chem., 262: 2869-2874 (1987); Noda et al., J. Biol. Chem., 263: 13916 (1988); Wrana et al., J. Cell. Biol., 106: 915 (1988); Noda et al., J. Cell. Physiol., 133: 426 (1987); Pfeilshifter et al., Endocrinology, 121: 212 (1987); Centrella et al., Endocrinology, 119: 2306 (1986); Roby et al., J. Cell. Biol., 105: 457 (1987). In other *in vitro* models, TGF-β was found to inhibit proliferation and expression of alkaline phosphatase and osteocalcin. See, for example, Noda and Rodan, Biochem. Biophys. Res. Commun., 140: 56 (1986); Noda, Endocrinology, 124: 612 (1989).

Further, while Centrella et al., *supra*, showed increased collagen synthesis after treatment of osteoblasts from rat calvaria with TGF-β, Robey et al., *supra*, could not show increased synthesis of collagen in fetal bovine bone osteoblasts, postulating that the increased collagen production is secondary to the effects of TGF-β on the proliferation of osteoblasts. In organ culture, TGF-β was reported to stimulate bone resorption in neonatal mouse calvarias, but inhibit resorption in the fetal rat long bone system. See Tashjian et al., Proc. Natl. Acad. Sci. USA, 82: 4535 (1981); Pfeilshifter et al., J. Clin. Invest., 82: 680 (1988). TGF-β activity was reported to be increased in cultures of fetal rat calvaria and in calvarial cells incubated with stimulators of bone resorption, such as parathyroid hormone, 1,25-dihydroxyvitamin D_3, and IL-1 (Petkovich et al., J. Biol. Chem., 262: 13424-13428 [1987], Pfeilschifter and Mundy, Proc. Natl. Acad. Sci. USA, 84: 2024-2028 [1987]). Furthermore, it was reported that TGF-β inhibits the formation of osteoclasts in bone marrow cultures. Chenu et al., Proc. Natl. Acad. Sci. USA, 85: 5683-5687 (1988). The showing that TGF-β has effects on both osteoclasts and osteoblasts led Pfeilschifter and Mundy, *supra*, to propose that it is involved in the strict coupling of the processes of bone resorption and bone formation characteristic of the remodeling process in adult bone. It has also been postulated that the local acidic, proteolytic environment provided by the osteoclasts results in activation of matrix-

associated latent TGF-β. Oreffo et al., Calcified Tiss. Internatl., 42: Suppl:A15 (1988).

In view of the conflicting results reported for *in vitro* activities, it is not clear whether *in vitro* models can be used to predict the effects of TGF-β on bone formation and resorption *in vivo*. See Roberts et al., Proc. Natl. Acad. Sci. USA, 82: 119 (1985).

Additional references reporting that TGF-β promotes the proliferation of connective and soft tissue for wound healing applications include U.S. Pat. No. 4,810,691 issued March 7, 1989, U.S. Pat. No. 4,774,228 issued September 27, 1988, Ignotz et al., J. Biol. Chem., 261: 4337 (1986); Varga et al., Biochem. Biophys. Res. Comm., 138: 974 (1986); Roberts et al., Proc. Natl. Acad. Sci. USA, 78: 5339 (1981); Roberts et al., Fed. Proc., 42: 2621 (1983); U.S. Pat. No. 4,774,228 to Seyedin et al. TGF-β stimulates the proliferation of epithelia (Matsui et al., Proc. Natl. Acad. Sci. USA, 83: 2438 [1986]; Shipley et al. Cancer Res., 46: 2068 [1986]); induces collagen secretion in human fibroblast cultures (Chua et al., J. Biol. Chem., 260: 5213-5216 [1983]); stimulates the release of prostaglandins and mobilization of calcium (Tashjian et al., Proc. Natl. Acad. Sci. USA, 82: 4535 [1985]); and inhibits endothelial regeneration (Heimark et al., Science, 233: 1078 [1986]).

In wound chambers implanted subcutaneously, TGF-β increased DNA and collagen production. Sporn et al., Science, 219: 1329 (1983); Sprugel et al., Am. J. Pathol., 129: 601 (1987). Moreover, TGF-β produced collagen fibrosis when injected subcutaneously (Roberts et al., Proc. Natl Acad. Sci. USA, 83: 4167-4171 [1986]) and promoted healing of skin incisions in rats (Mustoe et al., Science, 237: 1333 [1987]). Nevertheless, although TGF-β induced chondrogenesis in muscle-derived cells *in vitro* (Seyedin et al., Proc. Natl. Acad. Sci. USA, 82: 2267 [1985]; Seyedin et al., J. Biol. Chem., 261: 5693 [1986]), it did not produce cartilage *in vivo* even when implanted with collagenous substrates, a system used for a long time as a bone induction model in animals (Sampath et al., Proc. Natl. Acad. Sci. USA, 84: 7109 [1987]; Howes et al., Calcif. Tissue Int., 42: 34 [1988]).

New studies have shown a time-dependent appearance of mRNA for TGF-β1 at a fracture site in a rat and have localized the peptide immunohistochemically in the periosteum of the healing fracture; the same researchers reported that injections of TGF-β1 into the periosteal area of the femur of young rats have caused significant formation of new cartilage. Bolander et al., New York Academy of Sciences, "Transforming Growth Factor-βs: Chemistry, Biology and Therapeutics, May 18-20. 1989. It has been found that injections of TGF-β1 into the parietal bone of young rats stimulated periosteal bone formation, resulting in a thickening of the calvarium. Noda et al.. J. Cell. Biol., 107: 48 (1988).

TGF-β was reported to stimulate local periosteal woven bone formation when injected daily onto the periostea of parietal bones of neonatal rats. Noda and Camilliere, Endocrinology, 124: 2991-2994 (1989). The fact that TGF-β increases bone thickness when applied adjacent to periosteum *in vivo* is also reported in Joyce et al., J. Cell Biol., 110: 2195-2207 (1990); Marcelli et al., J. Bone Min. Res., 5: 1087-1096 (1990); Mackie et al., Bone, 11: 295-300 (1990).

Certain researchers reported that TGF-β does not induce bone formation unless it is administered concurrently with a cofactor, e.g., an osteoinductive factor purified from bovine demineralized bone. Bentz et al., *supra*, U.S. Pat. No. 4,843,063 issued June 27, 1989 to Seyedin et al., and U.S. Pat. No. 4,774,322 issued September 27, 1988.

The remodeling of bone with TGF-β is also described by Centrella et al., J. Bone and Jt. Surg., 73A: 1418-1428 (1991). Multiple applications of TGF-β1 to rat femur induced a profound stimulatory effect with increased deposition of bone at the site of injection. Joyce et al., J. Bone Min. Res., 4: 255-259 (1989). Additionally, a single local application of TGF-β1 in a methylcellulose gel formulation to sites of cartilage damage accelerated the onset and increased the incidence of bone formation adjacent to the cartilage. Beck et al., J. Bone and Mineral Research, 6: 961-968 (1991). A single local application of this same formulation in the rabbit skull defect model increased the amount of bone formation in a dose-dependent manner when measured 28 days after injury. Beck et al., J. Bone Min. Res., 6: 1257-1265 (1991).

Phosphate biomaterials have been prepared and investigated in a number of forms. The most widely studied are biodegradable beta tricalcium phosphate (TCP) and hydroxyapatite. A detailed description of the variety of calcium phosphate compositions studied can be found in deGroot, Bioceramics of Calcium Phosphate, Boca Raton. Florida, CRC Press, 1983. TCP is used as an *in vivo* scaffold for bone repair. Perhaps the most consistent and desirable property of TCP as well as other calcium phosphate ceramics is biocompatibility. Also, calcium phosphate ceramics are able to bond directly to bone. Driskell, Proc. Ann. Conf. Biomed. Eng., 15: 199 (1973).

While TCP has low impact resistance, it has application as a bone graft substitute or extender to the extent that proper fixation can be included during the TCP resorption and bone repair processes. It has been demonstrated that TCP in granular form can be used as an autogenous bone extender in the repair of long-bone discontinuities in rabbits. Lemons et al., First World Biomat. Cong. (Baden, Austria), 1980, 4.10.3 (Abstract). The surgically created defects filled with 50:50 TCP:autogenous bone healed in six weeks as compared with four to six weeks when autogenous bone alone was used. These results indicate that some applications of the granular TCP may be possible in humans where a degree of stress-bearing is a factor. Porous TCP has been applied in block form with some success in mandibular discontinuities in dogs. Tortorelli and Posey, J. Dent. Res., 60: Special Issue A:601 (1981) (abstract).

The principal clinical application of TCP has been in dentistry. Powdered TCP has been used for initiating apical closure in teeth and for treating periapical defects. Biodegradables may play a role as carriers for bone-inductive agents or bone-cell chemotactic factors. Dipolar microspheres or packets of osteoprogenitor cells donated by an individual may be incorporated within a polymer or ceramic, and in conjunction with characterized bone inductive proteins can be

expected to enhance bone repair and augmentation at any chosen skeletal site. Hollinger et al., Biodegradable Bone Repair Materials, 207: 290-305 (1986).

TGF-β is typically formulated at an acidic pH at which it is active. Various methods for its formulation include adding 2-5% methylcellulose to form a gel (Beck et al., Growth Factors, 3: 267-275 [1990] reporting the effects on wound healing of TGF-β in 3% methylcellulose), adding collagen to form an ointment or suspension (EP 105,014 published 4 April 1984; EP 243,179 published 28 October 1987; EP 213,776 published 11 March 1987), or adding a cosmetically acceptable vehicle to the TGF-β for a topical formulation (U.S. Pat. No. 5,037,643 issued 6 August 1991).

Additionally, human topical applications containing growth factors such as TGF-β are described in EP 261,599 published 30 March 1988. A slow-release composition of a carbohydrate polymer such as a cellulose and a protein such as a growth factor is disclosed in EP 193,917 published 10 September 1986. A formulation of a bioactive protein and a polysaccharide is described in GB Pat. No. 2,160,528 granted 9 March 1988. An intranasally applicable powdery pharmaceutical composition containing an active polypeptide, a quaternary ammonium compound, and a lower alkyl ether of cellulose is described in EP 193,372 published 3 September 1986. See also U.S. Pat. No, 4,609,640 issued 2 September 1986 disclosing a therapeutic agent and a water-soluble chelating agent selected from polysaccharides, celluloses, starches, dextroses, polypeptides, and synthetic polymers able to chelate Ca and Mg; and JP 57/026625 published 12 February 1982 disclosing a preparation of a protein and water-soluble polymer such as soluble cellulose. In addition, a method for entrapping enzymes in gel beads for use as a biocatalyst is described in U.S. Pat. No. 3,859,169. Also, a method for preparing polyvinyl alcohol gel intended as a transdermal vehicle for water-soluble synthetic drugs is disclosed in JP 62/205035 published 9 Sept. 1987.

A purified particulate bone mineral product for use in medicine impregnated with a gel-forming protein or polysaccharide such as gelatin is disclosed that may also carry one or more absorbed drugs such as transforming bone growth factor. WO 90/01955 published 8 March 1990. Use of TGF-β and a biocompatible controlled release polymer is described by Langer and Moses, J. Cell. Biochem., 45: 340-345 (1991). An osteoinductive pharmaceutical formulation comprising an anti-fibrinolytic agent such as epsilon amino acid caproic acid or other lysine analogue or serine protease inhibitor and a cartilage and/or bone inductive protein such as bone morphogenetic protein is disclosed in WO 91/19510 published 26 December 1991. The formulation may additionally contain a growth factor such as TGF-β and may be encased in a TCP matrix.

Biologically active polypeptides based on TGF-β sequences disclosed as useful in the treatment of wounds and bone fractures are described in WO 90/14359 published 29 November 1990. In addition, TGF-β has been disclosed as a treatment for gingivitis and periodontal disease in the form of implants, microspheres, an absorbable putty-like matrix, or a polymeric material having the drug impregnated thereon. WO 90/04974 published 17 May 1990. Compositions with activin, also optionally containing a TGF-β, a bone morphogenetic protein, or bone marrow, have been formulated with hydroxyapatite and TCP as a dental and orthopedic implant and for bone growth induction. WO 92/14481 published 3 September 1992, Also, TGF-β formulated for treatment of inflammatory disorders is described in EP 269,408 published 1 June 1988. Additionally disclosed are cytokines such as TGF-β bound to a solid support, which may include ceramics and polymeric materials as well as insoluble protein materials such as gelatin, collagen, or albumin. WO 90/09798 published 7 September 1990.

Stable lyophilized formulations of polypeptide growth factors such as TGF-β containing polymers to impart viscosity to a reconstituted solution or polysaccharides to stabilize against loss of biological activity are described in EP 308,238 published 22 March 1989 and EP 267,015 published 11 May 1988, respectively. See also EP 335,554 published 4 October 1989 on a cosmetic composition suitable for topical application to mammalian skin or hair that can contain collagen, a gelatin, and powders such as starch and aluminum silicates. Gels with polymeric material for providing viscosity that may contain a polypeptide growth factor such as TGF-β are described in EP 312,208 published 19 April 1989. Collagen-polymer conjugates in admixture with particulate matter such as TCP are described by WO 90/05755 published 31 May 1990. A controlled drug delivery system for placement in a periodontal pocket containing discrete microparticles comprising the drug (e.g., TGF-β) and a polymer is described in EP 451,390 published 16 October 1991. A bioactive compound associated with liposomes that may include TGF-β is described in EP 393,707 published 24 October 1990 and in Strassman *et al.*, Clin. Exp. Immunol., 86: 532-536 (1991).

A sustained-release formulation containing an active ingredient such as TGF and collagen and a least one organic acidic compound is described in EP 326,151 published 2 August 1989. TGF-β in combination with a proteinaceous matrix that may comprise collagen and/or fibrinogen is described by WO 91/03491 published 21 March 1991. A collagen sponge useful as an implant for a wound-healing matrix for TGF-β and FGF is described in U.S. Pat. No. 4,950,483 issued 21 August 1990. A therapeutic drug that contains a growth factor may be formulated in the form of powder, granules, etc., for example, with gelatin. JP 1-153647 published 15 June 1989. Cicatrising compositions containing activated TGF-β may be formulated with polysaccharides and humectants such as glycerol. FR 2,667,789 published 17 April 1992.

It has also been known to mix an active medicament unstable to heat with a biodegradable protein carrier such as collagen, atelocollagen, or gelatin to form a carrier matrix having sustained-release properties. The resultant mixture is then dried, and the dried material is formed into an appropriate shape, as described in U.S. Pat. No. 4,774,091.

It would be desirable to provide a formulation for TGF-β with the proper consistency suitable for molding to fill in bone gaps where needed.

Accordingly, it is an object of the present invention to provide a suitable formulation of exogenous TGF-β to a local site on an animal where skeletal (bony) tissue is deficient so as to produce in every case mature, morphologically normal bone at the site of administration where it is needed.

It is another object to provide a bone-inducing composition that is clinically relevant for filling in smaller bone defects than is required for prosthetic devices.

It is further object to provide a TGF-β formulation with enhanced consistency for improved application to the desired bone defect site.

These and other objects will become apparent to those skilled in the art.

Summary of the Invention

The above objects are achieved by providing a bone-inducing formulation comprising an effective amount of TGF-β and TCP. The TCP is in the form of particles. In a specific aspect, this formulation is a bone-inducing formulation comprising about 0.5 μg to about 5 mg TGF-β, more preferably 5 μg to about 3 mg TGF-β, adsorbed onto about 140 mg to about 50 g TCP particles, preferably granules.

In a preferred aspect, the formulation also contains an effective amount of a polymer for enhancing consistency of the formulation. More preferably, the polymer is amylopectin. In a specific aspect, this bone-inducing formulation comprises about 0.5 μg to about 5 mg TGF-β, about 140 mg to about 50 g TCP particles, and an amount of amylopectin that ranges from about 0.1:1 to 1:1 amylopectin:TCP, prefarably about 0.25:1 to 0.5:1 amylopectin:TCP.

In another aspect, the invention provides a method of producing a bone-inducing formulation of TGF-β comprising admixing an effective amount of a liquid solution of the TGF-β with TCP granules for a sufficient period of time to adsorb the TGF-β onto the granules and contacting the resulting mixture with an effective amount of amylopectin.

Formulations according to aspects of the present invention exclude activin and bone morphogenetic cofactor.

These aspects of the invention enable preparation of a suitable formulation for the generation of normal mature bone every time only where it is required at a particular site. Preclinical results with TGF-β applied topically as described below show new bone formation in various animal models.

Brief Description of the Drawings

Figure 1 illustrates the percentage of wounds with bone formation when placebo (left-most bar), recombinant human TGF-1 (rhTGF-β1) at 25 ng/wound (middle bar), or rhTGF-β1 at 100 ng/wound (right-most bar) is applied in the rabbit ear ulcer model at 42 and 70 days after wounding. Maximum bone formation was observed at day 42.

Figure 2 illustrates the non-defect end width, an indication of the efficacy in the rabbit skull defect model, on day 28 post administration of placebo and TCP discs with rhTGF-β adsorbed at two different concentrations, where * $p < 0.05$.

Figure 3 illustrates the adsorption kinetics of TGF-β in the presence of TCP granules (circles) and in the absence of TCP granules (squares).

Figure 4 discloses a graph of the amount of TGF-β adsorbed on TCP granules as a function of the concentration of TGF-β in the bathing solution.

Figure 5 illustrates the skull defect area in the rabbit skull defect model on day 28 post administration of placebo and TCP granules (40-100 mesh) with TGF-β adsorbed at two different concentrations, wherein * $p < 0.05$.

Figure 6 illustrates the skull defect area in the rabbit skull defect model on day 28 post administration of placebo and TCP (300 mg)/12% gelatin with TGF-β adsorbed at two different concentrations, wherein * $p < 0.01$.

Figure 7 illustrates the skull defect area in the rabbit skull defect model on day 28 post administration of placebo and TCP granules in lyophilized gelatin with TGF-β adsorbed at two different concentrations, wherein * $p < 0.05$.

Figure 8 illustrates total resorption surface in the rabbit skull defect model on day 28 post administration of a first lot of amylopectin with low endotoxin levels (1), a second lot of amylopectin with higher endotoxin levels (2), 5 μm TCP (3), amylopectin + 250 μm TCP (4), amylopectin + 10 μg TGF-β (5), amylopectin + 5 μm TCP + 10 μg TGF-β (6), amylopectin + 250 μm TCP + 10 μg TGF-β (7), and 10 μg TGF-β + 250 μm TCP + amylopectin (8).

Figure 9 illustrates the skull defect area in the rabbit skull defect model on day 28 post administration of formulations 1-8 defined in the legend to Figure 8, wherein * $p < 0.05$.

Figure 10 illustrates release over time of TGF-β from an amylopectin/TCP formulation as analyzed by ELISA, where the open circles are release into normal human serum and the solid circles are release into PBS/0.5% BSA.

Description of the Preferred Embodiments

A. Definitions:

The "polymer for enhancing consistency of the formulation" may be any polysaccharide or insoluble protein material useful for binding the TGF-β to the TCP to form a smooth, moldable putty or paste. Especially preferred are carbohydrates such as agarose, cross-linked agarose, dextran, cross-linked dextran, inulin, hyaluronic acid, cellulose, cellulose derivatives such as carboxymethyl cellulose, starch derivatives such as amylopectin, and insoluble protein materials such as gelatin, including lyophilized gelatin with glycerol, collagen, or albumin, or a combination of any of these. The collagen may be chemically conjugated to a synthetic hydrophilic polymer and mixed with the TCP as described in WO 90/05755, *supra*. The preferred polymer herein is amylopectin, most preferably potato amylopectin. Amylopectin is the branched component of starch; it is formed through chains of D-glucopyranose residues linked together mainly by (1-- > 4)-α-D linkages but with 5-6% of (1-- > 6)-α-D bonds at the branch points. It is further described in Molecular Biology, an International Series of Monograms and Textbooks, The Polysaccharides, Vol. 3, Gerald Aspinall, ed. (Academic Press, 1985), pp. 216-223.

"Tricalcium phosphate" or "TCP" has a nominal composition of $Ca_3(PO_4)_2$ and is found in two different whitlockite crystallographic configurations, α-TCP, and the more stable, β-TCP. It is an extremely biocompatible material used for filling bone and dental defects. It is described, for example, by Damien and Parsons, J. App. Biomaterials, 2: 187-208 (1991), Ricci, Biomedical Engineering: Recent Developments, Saha editor, "Development of a Fast-Setting Ceramics-Based Grout Material for Filling Bone," p. 475-481 (1986), Bowers et al., J. Periodontal, 57: 286-287 (1986). It has also been used with bone morphogenetic protein as a delivery system. Urist et al., Clin. Orthop., 187: 277-280 (1984). TCP is commercially available from, for example, DePuy, but also may be synthesized, for example, by the method described in Biomedical Sciences Instrumentation, Instrument Society of America, Ed. David Carlson, Vol. 27, Paper #91-026, Benghuzzi et al., p. 197-203 (1991). The preferred TCP herein is β-TCP, and in the examples below, the term "TCP" refers to β-TCP.

By "bone inducing" is meant promoting the formation of morphologically normal, mature bone only at a site where there is a bone deficiency that needs to be replaced. Mature bone is bone of any type, whether cortical or trabecular, that is mineralized as opposed to immature or cartilaginous bone as would be formed in a neonatal model. Morphologically normal bone is bone that is detected histologically as normal (i.e., consisting of endochondral or membranous type lamellar bone and including marrow spaces with osteoblasts and osteoclasts). This is in contrast, for example, to callous formation with a fibrotic matrix as seen in the first stage of fracture healing. Thus, the bone induction herein is contemplated not only as acceleration of bone regeneration, as in a fracture, but also as stimulation of the formation of bone that is returned to its normal morphological state.

By "skeletal tissue deficiency" is meant a deficiency in bone at any site where it is desired to restore the bone, no matter how bone deficiency originated, e.g., whether as a result of surgical intervention, removal of tumor, ulceration, implant, or fracture.

By "bone morphogenetic cofactor" is meant a protein originally found in the bone matrix that induces all of the cascade events involved in the osteoinductive process *in vivo*, including chondrogenesis, vascular invasion, formation of a marrow cavity, and eventually formation of a bone ossicle. Such factors include the bone morphogenetic proteins as found in demineralized bone (Urist, Science, 150: 893 [1965]), osteogenin, a 22 Kd protein with this activity (Sampath et al., Proc. Natl. Acad. Sci. USA, 84: 7109 [1987]), a glycoprotein called osteoinductive factor (U.S. Pat. No, 4,843,063, *supra*), and BMP-1, BMP-2A, and BMP-3 from demineralized ovine bone matrix (Wang et al, Proc. Natl. Acad. Sci. USA, 85: 9484 [1988]; Wozney et al., Science, 242: 1528 [1988]).

The osteoinductive cofactor described in the U.S. patent is isolated from bone, preferably a bovine metatarsal bone, wherein the demineralized bone is prepared, non-collagenous proteins are extracted from the bone, the extract is subjected to gel filtration, the fraction constituting a low molecular weight (10,000-40,000 daltons) possessing the greatest chondrogenic activity is subjected to ion exchange chromatography, the first fraction CM-1 is subjected to RP-HPLC, and two peaks of predominantly 28 Kd and 36 Kd chondrogenic/osteogenic cofactor protein are purified to give single bands on SDS-PAGE. These cofactors and the others mentioned above are included in the term "bone morphogenetic cofactor."

By "osteogenic cell source" is meant a source of viable cells that are capable of forming bone, as well as viable cells that are precursors to cells capable of forming bone, including a source of cells capable of recruiting or stimulating cells capable of forming bone. Suitable such sources include dispersed whole bone marrow cells (obtained by, e.g., aspiration or mechanical agitation), perichondrium, periosteum, or a suitable cell line. For example, the cells may be taken from a site of the animal to be treated adjacent to the deficiency (e.g., periosteum stripped from an adjacent site to the defect such as a fracture site or a surgical excision site) or from a biopsy site of the animal (e.g., one that has been previously accessed, e.g., the hip), or from bone marrow.

By "animal" is meant any animal having a vertebrate structure, preferably a mammal, and most preferably a human.

By "TGF-β" is meant the family of molecules described hereinabove that have either the full-length, native amino

acid sequence of any of the TGF-βs from any species, including the latent forms and associated or unassociated complex of precursor and mature TGF-β ("latent TGF-β"). Reference to such TGF-β herein will be understood to be a reference to any one of the currently identified forms, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, and TGF-β5, each of which is represented by certain species indicated in Figure 1 of U.S. Pat. No. 5,158,934 issued October 27, 1992 and latent versions thereof, as well as to TGF-β species identified in the future, including polypeptides derived from the sequence of any known TGF-β and being at least 75% homologous with the sequence. Members of the TGF-β family are defined as those which have nine cysteine residues in the mature portion of the molecule, share at least 65% homology with other known TGF-β sequences in the mature region, and compete for the same receptor. In addition, they all appear to be encoded as a larger precursor that shares a region of high homology near the N-terminus and shows conservation of three cysteine residues in the portion of the precursor that will later be removed by processing. Moreover, the TGF-βs appear to have a four- or five-amino-acid processing site.

B. Modes for Carrying Out the Invention:

The invention is carried out in one aspect by mixing the TGF-β with tricalcium phosphate particles or granules, and without the bone morphogenetic cofactor and activin, and administering the resulting composition locally to a site on an animal where it is desired to induce formation of normal, adult bone and where a source of osteogenic cells and their precursor cells are present at the site. If the site does not naturally have a source of osteogenic cells present, the pharmaceutical composition also contains an osteogenic cell source as defined above, in an amount sufficient to induce bone growth.

Examples of indications where promotion of bone repair at a skeletal site is important include periodontal disease where root socket healing is impaired (tooth socket sites), non-union fractures, including primary treatment of high risk fractures and adjunctive treatment with bone grafting or bone substitutes for established non-union fractures, large bony defects caused by trauma or surgery [e.g., partial mandibular resection for cancer, large cranial defects, spinal (vertebral) fusions, correction of severe scoliosis by surgical alignment held in place with a Harrington bar (to shorten the six months normally required for a body cast), and spinal fractures with open reduction (to decrease significantly the period of immobilization)], and rapid stabilization and enhanced fixation of artificial prostheses and spacer bars, oral joints, and bone replacements.

Examples of the latter include plastic and reconstructive surgery, fixation of permanent dentures into mandible, enhanced fixation of accepted joint prosthesis, e.g., hips, knees, and shoulders (leading to the acceptance of prostheses that until now have been unacceptable due to rapid loosening and instability such as elbows), and limb salvage procedures, usually associated with malignancy (the bone shaft may be removed but the articular surfaces are left in place and connected by a space bar; rapid and enhanced fixation is required for success). If the site constitutes a periodontal site, i.e., one that involves the teeth, gums, and dental sockets, the TGF-β is suitably administered in conjunction with an exogenously added source of osteogenic cells.

In one preferred embodiment, the TGF-β is administered by treating a device with the TGF-β composition and implanting the device into the animal at the site of the deficiency, the composition also containing the osteogenic cell source when the site is deficient in such cells. The device may consist of any device suitable for implantation, including a molded implant, plug, prosthetic device, capsule, titanium alloy, sponge, or ceramic block. Examples of suitable delivery vehicles useful as devices are those disclosed by Nade et al., Clin. Orthop. Rel. Res., 181: 255-263 (1982); Uchida et al., J. Biomed. Mat. Res., 21: 1-10 (1987); Friedenstein et al., Exp. Hematol., 10: 217-227 (1982); Deporter et al., Calcif. Tissue Int., 42: 321-325 (1988); McDavid et al., J. Dent. Res., 58; 478-483 (1979); Ohgushi et al., J. Orthopaedic Res., 7: 568-578 (1989); Aprahamian et al., J. Biomed. Mat. Res., 21: 965-977 (1986); Emmanual et al., Stain. Tech., 62: 401-409 (1987).

Preferably, the device is treated with the TGF-β composition (which includes both a solution and a gel formulation) for a sufficient period of time to allow adsorption, and to allow drying in the case of the gel. The concentration of TGF-β in the solution or gel and the time of exposure depend on a number of factors, including the volume of the defect, the potency of the TGF-β polypeptide, and the nature of the site to which it is applied, and will be adjusted accordingly. As the size of the defect increases, or when the site is other than a bone site, the concentration of TGF-β and the time of presoaking should be increased. The treatment is for preferably at least about 0.5 hour, depending on the factors mentioned above (more preferably at least about 1 hour, and most preferably 1-2 hours), before implantation. Also depending on the above considerations, the concentration of TGF-β in the TGF-β composition for treating the device is preferably at least about 1 ng/ml (more preferably at least about 1-10 up to 100 ng/ml). The treatment may consist of any mode by which the composition is applied to the device to deliver effectively the TGF-β and the osteogenic cell source. Such treatment includes, for example, adsorption, covalent crosslinking, or impregnation, depending in part on the nature of the indication.

The TGF-β compositions to be used in the therapy will be dosed in a fashion consistent with good medical practice taking into account the nature of the skeletal tissue deficiency to be treated, the species of the host, the medical condition of the individual patient, the presence of any other cotreatment drug in the composition, the site of delivery of the

agent, the method of administration, the scheduling of administration, and other factors known to practitioners. Because of differences in host response, significant site-to-site and patient-to-patient variability exists. For purposes herein, the "therapeutically effective amount" of TGF-β is an amount that is effective to induce bone growth, as defined above, at the site of skeletal tissue deficiency.

As a general proposition, the TGF-β is formulated and delivered to the target site at a dosage capable of establishing at the site a TGF-β level greater than about 0.1 ng/ml. Typically, the TGF-β concentrations range from about 0.1 ng/ml to 5 mg/ml, preferably from about 1 to 2000 ng/ml. These intra-tissue concentrations are maintained preferably by topical application and/or sustained release.

As noted above, these suggested amounts of TGF-β are subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose and scheduling is the result obtained. Clinical parameters to determine an endpoint include increases in bone formation and mass and increases in radiographically detectable bone. Such measurements are well known to those clinicians and pharmacologists skilled in the art. The TGF-β composition is administered locally to the site by any suitable means, including topical and continuous-release formulation. The active TGF-β ingredient is generally combined at ambient temperature at the appropriate pH, and at the desired degree of purity, with a physiologically acceptable carrier, i.e., a carrier that is non-toxic to the patient at the dosages and concentrations employed. The carrier may take a wide variety of forms depending on the form of preparation desired for administration.

To be effective, the TGF-β is converted by the body to its activated form, i.e., the mature form is cleaved from its precursor using a suitable enzyme and the resultant complex is treated with acid or other appropriate agent to activate the TGF-β. Nevertheless, TGF-β is suitably administered in an inactive or delayed-release form such as a complex of mature TGF-β with proTGF-β not containing mature TGF-β (i.e., the remaining precursor of TGF-β), with a TGF-β binding protein, or with alpha$_2$-macroglobulin. The latent form is then converted to the active form either by naturally occurring mechanisms in the local environment or by formulation with TGF-β activating agents described above. See, e.g., Gentry et al., Mol. Cell. Biol., 8: 4162-4168 (1988); Miyazono et al., J. Biol. Chem., 263: 6407-6415 (1988); Wakefield et al., J. Biol. Chem., 263: 7646-7654 (1988); Keski-Oja et al., J. Cell Biochem. Suppl., 11A: 60 (1987); Kryceve-Martinerie et al., Int. J. Cancer, 35:553-558 (1985); Lawrence et al., Biochem. Biophys. Res. Commun., 133: 1026-1034 (1985); Lawrence et al., J. Cell Physiol., 121: 184-188 (1984). Thus, the pH of the TGF-β composition may suitably reflect the conditions necessary for activation.

For the preparation of a liquid composition suitable for impregnation of a device, the carrier is suitably a buffer, a low molecular weight (less than about 10 residues) polypeptide, a protein, an amino acid, a carbohydrate including glucose or dextrans, a chelating agent such as EDTA, a cellulose, or other excipient. In addition, the TGF-β composition is preferably sterile. Sterility is readily accomplished by sterile filtration through (0.2 micron) membranes TGF-β ordinarily will be stored as an aqueous solution, as it is highly stable to thermal and oxidative denaturation, although lyophilized formulations for reconstitution are acceptable.

Generally, where the bone disorder permits, one should formulate and dose the TGF-β for site-specific delivery, where the TGF-β is formulated into a sterile composition suitable for local application to the desired site.

For local application of the TGF-β composition, for example, in the case of a bone defect that is a crack, e.g., a union fracture, the carrier may be any vehicle effective for this purpose.

Delivery of TGF-β to the bony site is by way of TCP particles, which encompass, for example, granules and powder. While the particles generally can be any size, the preferred particle size of TCP in this invention is > 5 μm, more preferably greater than or equal to about 75 μm. More preferably, the size of the TCP granules is about 120-420 μm, most preferably about 125-250 μm, to obtain a granular putty that can be applied to defects that are not so wide as to require implants. The TGF-β is typically adsorbed onto the TCP.

The amount of TCP employed will depend mainly on the type of mammal being treated and the size of the defect. In humans, the amount of TCP could reach up to about 50 g. The amount of TGF-β would increase proportionately to TCP. Generally, the amounts range from about 0.5 μg to about 5 mg TGF-β, preferably about 1 μg to about 3 mg TGF-β, more preferably about 5 μg to about 1 mg TGF-β, adsorbed onto about 140 mg to about 50 g TCP particles, preferably granules. The amount of TGF-β will be adjusted downward in accordance with conventional clinical parameters if there is a biphasic response in which the efficacy of the TGF-β decreases with increasing TGF-β concentration for the same size defect.

Optionally the formulation of TGF-β and TCP also contains a polymer designed to bind the components together to improve consistency and ability to mold the resultant putty- or paste-like material. Examples of such polymers include, but are not limited to, amylopectin, gelatin, collagen, agarose, dextran, or a mixture of any two or more of these polymers. Further, the formulation suitably comprises the polymer in conjunction with a co-solvent such as glycerol, for example, gelatin and glycerol if the formulation is to be lyophilized before contact with the TCP and TGF-β mixture.

The polymer is present in the composition in an amount that depends mainly on the size of the TCP particles being employed, as well as on the type of polymer utilized and the amount of TGF-β and TCP used.

The TGF-β and TCP may be first mixed before exposure to the polymer, or they may all be mixed together at the same time, or the TGF-β may be mixed with the polymer and then with TCP. In a preferred mode, the TGF-β and TCP are first mixed before the polymer is used to bind the mixture.

A particularly preferred binding polymer herein is amylopectin, especially in combination with TCP granules. The method of preparation of the amylopectin/TCP formulation, and possibly other TCP formulations, can be dependent on the size of the TCP particles employed. Thus, for example, if the size of the TCP particles is less than about 100 μm, the ingredients may be contacted in any order, including simultaneously mixing the TGF-β with the amylopectin and TCP or adding the TCP to the amylopectin followed by the TGF-β. However, if the size of the TCP granules is greater than about 100 μm, the order of mixing ingredients may affect the efficacy, at least in one animal model, and thus a preferred method of producing a bone-inducing formulation of TGF-β for all sizes of TCP granules, and particularly for larger sizes, comprises admixing an effective amount of a liquid solution of the TGF-β with the TCP granules for a sufficient period of time to adsorb the TGF-β onto the granules and contacting the resulting mixture with an effective amount of amylopectin. Conditions that ensure adsorption of the TGF-β on the TCP particles are exposing the TCP to the TGF-β at a temperature above about 0°C, preferably at least about 5°C, more preferably about 5-40°C, still more preferably about 5-30°C, and most preferably about room temperature. The time of exposure to TGF-β is preferably not less than about 5 minutes, although shorter times may be possible. Then the amylopectin is added and mixed manually with the powder to homogeneity.

A preferred composition comprises about 0.5 μg to 5 mg TGF-β, about 140 μg to 50 g TCP particles, preferably granules, and an amount of amylopectin that ranges from about 0.1:1 to about 1:1 (weight/weight) amylopectin:TCP, preferably 0.25:1 to 0.5:1 amylopectin:TCP, depending on the size of the TCP particles. Thus, if the TCP particles are less than 5 μm, the ratio of amylopectin to TCP is preferably about 0.25 to 1, and if the TCP particles are greater than or equal to 75 or 125 μm, the ratio of amylopectin to TCP is preferably about 0.5 to 1, and the ratio of TCP:amylopectin:TGF-β solution is most preferably 1:0.5:0.5.

The amylopectin may be obtained from any source of starch, such as corn and potato, with potato being preferred. The amylopectin is preferably sterilized before use, as by autoclave or irradiation. To minimize the number of colony forming units (CFU) the amylopectin is suitably dissolved in water to form a solution of about 2-4% and then sterilized by autoclave (about 100-120°C for no less than about 30 minutes). To remove all the water, it is also preferably lyophilized or spray dried.

The composition herein also may suitably contain other peptide growth factors such as IGF-I, TGF-α, human growth hormone, epidermal growth factor, and PDGF, provided that such factors do not include the bone morphogenetic factors defined above. Such growth factors are suitably present in an amount that is effective for the purpose intended, i.e., to promote formation of bone.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention.

EXAMPLE 1

The TGF-β1 used herein was the recombinant expression product of transfected human 293 cells as described by EP 200,341, *supra*, and by Derynck et al., Nature, *supra*, and purified as described in Derynck et al., Nature, *supra*. The individual samples of recombinant human TGF-β1 (rhTGF-β1) were sterilely prepared in methylcellulose containing 20 mM sodium acetate buffer at pH 5.0 and applied as a single topical dose. Selected concentrations of rhTGF-β1 were mixed with methylcellulose gel so that the final concentration of methylcellulose was 3%. The vehicle was formulated in a similar manner without rhTGF-β1 as a control. The material was stored at 5°C until use.

The rat incisional model utilized young adult Simonsen Albino rats (300-350 g). Full thickness skin incisions were made by cutting through the subdermal panniculus carnosus musculature following application of Betadine™ brand antiseptic and 70% alcohol scrubbing to disinfect the surgical site. Two pairs of symmetrical transverse incisions (approximately 2.5 cm) were placed in each animal. A single dose of rhTGF-β1 in methylcellulose was placed into each stainless steel sutured wound by inserting a 25-gauge needle along the edge of the wound and below the sutures. The volume of rhTGF-β1 in 3% methylcellulose placed into each wound was 0.05 ml. Each rat had two incisions into which rhTGF-β1 in 3% methylcellulose was applied. One incision received either vehicle alone (3% methylcellulose) or no treatment at all. Concentrations of rhTGF-β1 were 500, 1000, 2000, or 4000 ng/ml. Dose response curves were developed using dose ranges of 5 to 10,000 ng/wound. Animals were euthanized on day 5, 7, 10, 14, 21, and 28. The entire dorsal skin was excised after the sutures were removed. Two 8-mm wide strips of skin were collected from each incision and fixed in 10% neutral buffered formalin for seven days.

New Zealand white male rabbits (2.5-2.8 kg) were purchased from Elkhorn rabbitry. Anesthesia was induced by an intramuscular injection of ketamine hydrochloride/xylazine hydrochloride mixture. After removal of hair from the ears, the area of the wound was sterilely prepared using Betadine™ brand antiseptic with an alcohol rinse. A circular 6-mm punch biopsy instrument was used to produce wounds to the depth of the ear cartilage. The underlying perichondrium was removed with a periosteal elevator and fine scissors. Wounds were treated with 0.025 ml of 3% methylcellulose or 5, 15, 25, 100, 500, or 1000 ng of rhTGF-β1 in 3% methylcellulose (control). Opsite™ surgical dressing was placed over each wound. An Elizabethian collar was placed around the neck of the rabbits to prevent mechanical disruption of the wounds by the rabbit.

Studies were also designed to examine short-term and long-term effects of topical rhTGF-β1. Wounds were harvested on days 3, 5, 7, 14, 21, 28, 42, 56, and 70. Wounds were photographed, cut into hemisections, and fixed in 10% neutral buffered formalin for histology and morphometric analysis. Morphometric analysis included measurements of total healing wound area, closing wound area, upper wound gap, lower wound gap, area of collagen, area of granulation tissue, epithelial cell layer length, and bone formation. These measurements were made on a BioQuant IV™ (R & M Biometrics Inc., Nashville, TN) computer image analysis system.

The rabbit ear ulcers were examined for delayed effects of rhTGF-β1 on days 21, 28, 42, 56, and 70 following a single application of 25 or 100 ng/wound on the day of wounding. Bone formation was observed along the wound edges and immediately adjacent to the cartilage. The bone was normal in morphological appearance, consisting of endochondral or membranous type bone and ossification with marrow spaces. Osteoblasts and osteoclasts were present. The percentage of wounds with bone increased to a maximum of 74% of the treated wounds at day 42 (100 ng/wound) and decreased to 69% by day 70. See Figure 1. Bone formation was observed in less than 12% of placebo-treated wounds.

No bone formation was observed in the rat incision model, indicating that bone formation is induced only at a site that has a source of precursor (osteogenic) cells, in this case in the rabbit ear model where the wound was adjacent to perichondrium.

EXAMPLE 2

A rat femur gap model was employed wherein a polyethylene plate 2-mm thick, 8-10 mm long, and 4-5 mm wide was pinned to one face of a rat femur with stainless steel pins. From the center of the femur a 5-8-mm long piece of bone was removed. The plate serves to keep the gap in bone separated. This model is intended to mimic a non-union fracture in a human.

Set into the gap in the femur is a porous cylindrical 200-to 400-micron ceramic implant of 60% by weight hydroxyapatite and 40% by weight TCP (Zimmer, Inc.), which is either (1) implant alone, (2) implant presoaked for 1 hour in a solution of 50 ng/ml rhTGF-β1 prepared as described in Example 1 and formulated in Delbecco's medium without serum, (3) implant plus dispersed whole bone marrow cells obtained from syngeneic rat, and (4) implant plus dispersed whole bone marrow cells pretreated with 50 ng/ml of the rhTGF-β1 in Delbecco's medium described above. A total of 15 rats were used for each of these four groups. One month after implant, the rats were sacrificed and analyzed for histological changes.

Preliminary results indicate that no bone replacement was observed in the control without cells or rhTGF-β nor with rhTGF-β without cells; TGF-β with cells was found to accelerate the rate of bone growth over cells alone. The bone formed with rhTGF-β was found in the interstices of the pores in the ceramic and bridged the gap. The bone formed with the rhTGF-β was found to be histologically normal.

EXAMPLE 3

A case study was performed using baboons to investigate the effect of TGF-β on bone wound healing. The baboon was selected because of the close analogy of its bone kinetics to those of man. A methylcellulose gel of TGF-β1 was delivered via an analytical bone implant, and after 22 days the implant was removed from the baboon. Tissue obtained from TGF-β implant sites was analyzed using quantitative histomorphometry to determine the mean effect of TGF-β on bone wound healing. Detailed non-quantitative histopathologic evaluation was also performed.

More specifically, four male baboons were implanted with four titanium analytical bone implants (cages) each, two per tibia in areas of close structural geometry. Holes were drilled in the tibia to allow implantation. After implantation, the baboons were allowed to heal for 41 days. On the 41st day, all the implant sites were surgically exposed, tissue was removed, and the test materials were implanted into the implant cores. Each animal received a normal (no treatment) control, a control with only methylcellulose vehicle, and a low (1 μg rhTGF-β in methylcellulose) or high (10 μg rhTGF-β in methylcellulose) dosage of active TGF-β. Specifically, these formulations each consisted of 1 g of 3.0% methylcellulose by weight, lactic acid QS to pH 5.0, and 0, 20, or 200 μg/ml of rhTGF-β1 prepared as described in Example 1. The formulations were poured into size 5 gelatin capsules (Elanco), which were then placed in the core of the titanium implant and used to deliver 50 μl of each formulation, with slow dissolution of the capsule. All implant sites within an animal were randomly assigned to one of the four treatments.

Following 22 days of healing, tissue in all implants was retrieved. The tissue samples were placed in 10% formalin solution, buffered to a pH of 7.0, containing formaldehyde at 3.7% for fixation. Samples were submitted for histopathologic analysis.

The following descriptive and quantitative observations were made:

1. Bone volume in TGF-β sites was lower than control and placebo sites, although not statistically significant.
2. Osteoblast numbers, volume, and activity were significantly greater in the TGF-β sites when compared to either the control or placebo.

3. Osteoclast numbers and activity appeared higher in all four treatment sites when subjectively compared to control data obtained in previous studies.

4. Residual methylcellulose was noted and appeared to require phagocytosis before new trabecular bone could form.

5. TGF-β in the presence of methylcellulose matrix was associated with increased numbers of fibroblast, osteoprogenitor cells, and osteoblasts.

6. No foreign body response or other adverse pathologic reaction to either matrix alone or matrix and TGF-β was observed.

7. Significant periosteal new bone formation was noted over the implants in five TGF-β sites in three animals. Bone formation over the implant to this degree had never been observed in over 450 titanium implant procedures carried out over the past few years.

8. TGF-β sites were identified during blinded histologic review in seven out of a total of eight sites.

9. Methylcellulose sites were identified during blinded histologic review 100% of the time.

Control samples analyzed in this study demonstrated that cancellous tissue formed in the titanium implant is stratified from inferior to superior aspects of the implant core. The superior portion of the tissue (closest to the cap of the titanium implant) is less mature and shows greater osteoblastic activity, while tissue near the inferior aspects of the implant and deep within the medullary compartment is more mature in morphology and shows a reduced osteoblastic population and activity. In contrast to historical and control samples, the TGF-β tissue samples were homogeneous in their high osteoblastic activity throughout the specimen.

Clinical observations of the tissue above and around the supra-periosteal portion of the titanium implant revealed pronounced periosteal bone formation. This periosteal bone formed large masses over two sites in each of two animals. The masses in these two animals were highly vascularized, had the clinical appearance of trabecular bone, and varied in size within one animal. The two masses in each of two animals were approximately 3x2x1.5 cm and 1.5x1x0.5 cm in size. One additional animal demonstrated pronounced periosteal bone formation over one TGF-β site. It is significant that in over 450 titanium implant surgical procedures masses like these have never formed over the titanium implants. Histologically, this periosteal bone formation over five TGF-β sites in three baboons was similar to an actively healing, uncomplicated, fracture callus, i.e., morphologically normal, mature bone formation.

In general, the methylcellulose was well tolerated and no foreign body response was present in any of the four treatment sites. Additionally, no evidence of cytologic atypia or malignancy was found in either titanium implants or periosteal samples.

EXAMPLE 4

Introduction

The purpose of this study was to evaluate the effects of TGF-β1 in the rabbit skull defect model of bone formation when incorporated into a TCP matrix that was configured as a thin disc the approximate size of the defect (12 mm). This was accomplished by measuring selected bone morphometric parameters from stained histologic sections as well as by radiographic examination of the excised defect site. Results were compared to defects administered TCP discs without TGF-β1.

Source and Preparation of TGF-β1 and TCP Discs

The rhTGF-β1 was prepared and purified as described in Example 1. Individual samples of the active portion of rhTGF-β1 were prepared under sterile conditions in 20 mM sodium acetate buffer at pH 5.0. The incorporation of rhTGF-β1 into TCP discs (obtained from DePuy, Warsaw, Indiana) was done by aseptically incubating TCP in the TGF-β1 solution for three hours at room temperature. Prior to the incubation, TCP discs were sterilized by incubating in 70% ethanol, rinsing thoroughly with sterile normal saline, and drying under UV lamp. The average weight of each disc was 153 mg. Two different concentrations of rhTGF-β1 were used, 20 and 100 μg/ml. After incubation, each disc was rinsed briefly with sterile normal saline. The amount of rhTGF-β1 adsorbed onto the TCP disc was determined from the changes in the concentration of TGF-β1 incubating solutions by conventional ELISA methods. The higher concentration (100 μg/ml) gave the average value of 16 μg/disc, while 5 μg/disc was the average value from the incubation of the discs with 20 μg/ml TGF-β1.

Animal Surgery and Treatment

All studies were performed in accordance with the American Association for the Accreditation of Laboratory Animal Care (AAALAC) guidelines. Sixteen male New Zealand White rabbits (2.8 - 3.2 kg) (Elkhorn Rabbitry, Watsonville, CA)

were anesthetized with 0.75 ml/kg Hypnorm® brand anesthesia (Jenssen Pharmaceutica, Beersa, Belgium). The top of the head and base of the ears were shaved and aseptically prepared for surgery. An elliptical incision was made over the skull, reflecting the skin flap anteriorly. Similarly, the periosteum was reflected anteriorly as a flap, exposing the top of the skull. Both skin and periosteal flaps were covered with sterile, moistened gauze. A 12-mm skull defect was selected since, in the absence of treatment, bone does not bridge the gap, but rather a fibrous tissue non-union of the skull persists. Frame, J. Oral Surg., 38: 176-180 (1980). A sterile trephine attached to an electric drill was used to produce the defect at the point of intersection between the sutures of the right and left parietal and frontal bones. The site was liberally irrigated with physiological saline during the drilling to prevent overheating of the bone margins. Care was taken not to puncture or damage the underlying dura. A precut, sterile saline-moistened piece of Gelfilm™ brand of film (Upjohn, Kalamazoo, MI) was inserted through the defect overlying the dura to function as a barrier between the dura and the edges of bone.

Sterile TCP discs or TCP discs with rhTGF-β1 (5 or 16 μg) were applied to the defect filling the defect. The periosteal flap was sutured back in place with 6-0 proline sutures and the skin flap was closed with 4-0 silk. Rabbits were returned to their cages and allowed to recover. After 28 days rabbits were euthanized with an overdose of barbiturate and the defect sites were removed with adjacent normal bone. The defect sites were rinsed in physiological saline. Sites were fixed in 10% neutral buffered formalin and radiographed using a Faxitron™ brand X-ray system and X-omat AR-2 film exposed at 25 KV, 10 s. The fixed tissue samples were then cut in half at the center of the defect parallel to the frontal/parietal suture. One hemisection was acid decalcified (Easy-cut™ reagent, American Histology Reagent Co., Modesto, CA) and processed by routine histologic methods using hematoxylin and eosin to stain the 4-μm sections. The other half of the defect was plastic embedded, and undecalcified sections were processed by routine histologic methods, with the 5-μm sections stained with Goldners' trichrome, von kossa, or toluidine blue.

Goldner's trichrome stained sections were examined using a BioQuant IV™ computer image analysis system. Selected indices of bone formation and resorption were measured, including trabecular bone volume (TBV), percentage osteoid surface (%OS), percentage osteoid volume (%OV), mean osteoid width (OW), percentage osteoblast/osteoid (%Ob/Ost), percentage osteoblast/total surface (%Ob/TS), total resorption surface (TRS), and number (#) of osteoclasts/surface length (Oc/SL). Sections from all animals were analyzed histomorphometrically using a random stratified sampling scheme that systematically evaluated selected fields from the bony edge of the defect and the entire area within the defect. Fields were selected using a grid pattern, such that each field within the defect area had an equal probability of being selected. Approximately equal numbers of fields were evaluated for both the control and treated defects.

The thickness (width) of bone at the outside edge of the sections (at the edge of the harvested sample farthest from the detect site) was measured to evaluate the extent of bone formation at non-defect sites (non-defect end width, NEDW). Defect area that is normally quantitated radiographically using computer image analysis could not be determined accurately due to the radiopaque nature of the TCP discs.

Statistical analysis

Data were analyzed by one factor ANOVA and the Scheffe F-test to determine differences between groups. The test of significance was performed at the 95% confidence interval compared to vehicle control. Each group contained three to four rabbits.

Results

Morphometric Evaluation. Data from the morphometric determinations are presented in Table 1. In general, TGF-β1-impregnated TCP discs stimulated a greater degree of bone formation at the defect site compared to TCP discs without TGF-β1. Indices of bone formation, including trabecular bone volume, osteoid width, osteoid volume, and osteoblast/osteoid were increased in the TGF-β1-treated defects compared to vehicle-treated defects. In addition, the number of osteoclasts/surface length and total resorption surface were increased in TGF-β1-treated defects compared to vehicle-treated defects, indicating that remodeling processes were present. Non-defect end width of bone from defects administered either 5 or 16 μg of TGF-β1 were greater than placebo-treated defects (see Table 1 and Fig. 2). The only parameters without significance between groups were osteoid surface and osteoblast/total surface.

TABLE 1

Histomorphometric Evaluation of Bone Formation in Skull Defects Applied TGF-β1 Impregnated onto TCP Discs[a]			
Histomorphometric Parameters[b]	TGF-β		
	Vehicle	5 μg	16 μg
TBV (%)	1.64±0.83	21.60±3.43[c]	15.59±2.75[c]
OW (mm)	0.003±0.003	0.01±0.001[d]	0.009±0.001[d]
OV (%)	0.76±0.76	9.07±2.17[c]	9.19±0.73[c]
OS (%)	27.68±27.68	46.59±6.42	42.81±4.30
Ob/TS (%)	36.44±31.99	56.86±5.04	65.69±1.39
Ob/Ost (%)	40.14±40.14	125.43±8.56[d]	157.99±15.34[d]
Oc/SL (#)	0±0	0.53±0.09[d]	0.57±0.20[d]
TRS (%)	0±0	0.025±0.005[d]	0.027±0.009[d]
NDEW (mm)	1.37±0.11	1.69±0.04[d]	1.75±0.11[d]

[a] Values reported are mean ± S.E.M. based. N = 3 for vehicle and 4 each for 5 or 16 μg of TGF-β1.
[b] TBV is trabecular bone volume; OW is osteoid width, OV is osteoid volume, OS is osteoid surface, Ob/TS is osteoblast/total surface, Ob/Ost is osteoblast/osteoid, Oc/SL is osteoclasts/surface length, TRS is total resorption surface, and NDEW is non-defect end width.
[c] $p < 0.01$.
[d] $p < 0.05$.

Due to the radiodense nature of the TCP discs, radiographic defect area was not determined. While there were apparent differences between placebo-treated and TGF-β1-treated defects, these differences were not amenable to morphometric determinations. However, the TGF-β1-treated defects appeared slightly more radiopaque, with the defect area and non-defect area blending without sharp border between the edge of the TCP disc and skull.

Histological evaluation. Histologic evaluation of the defects filled with TCP discs impregnated with TGF-β1 indicated an increase in the amount of bone surrounding the TCP disc. In addition, bone was observed migrating into the surfaces of the disc primarily at the margin of the defect, but also on the top and bottom surfaces. The new bone was characterized as a mixture of woven (immature) and lamellar (mature) bone by polarized light microscopic examination. In contrast, a minimal bony response was observed histologically in the TCP discs without TGF-β1.

In summary, the TCP discs impregnated with TGF-β1 induced a marked increase in bone both surrounding the discs as well as migrating into the discs. Bone was characterized histologically as a mixture of immature and mature bone indicating active formation and resorption processes. Remodeling of bone was subsequently confirmed histomorphometrically by an increase in both formation and resorption parameters within TGF-β1-treated sites. TCP discs without TGF-β1 were minimally inductive at 28 days with only slight amounts of bone located at the margins of the defect.

These data demonstrate that TCP will function as a carrier for TGF-β1 and provide a matrix on which bone can readily form across osseous defects.

EXAMPLE 5

Introduction

The purpose of this study was to evaluate the effects of TGF-β1 in the rabbit skull defect model of bone formation when incorporated into 40-100 mesh TCP matrix, wherein the TCP is supplied as granules. This was accomplished by measuring selected bone morphometric parameters from stained histologic sections as well as by radiographic examination of the excised defect site. Results were compared to defects administered 40-100 mesh TCP without TGF-β1.

Source and Preparation of TGF-β and TCP Matrix

rhTGF-β1 was prepared as described in Example 1. Individual samples of the active portion of rhTGF-β1 were prepared under sterile conditions in 20 mM sodium acetate buffer at pH 5.0. Two different concentrations of rhTGF-β1 were used, 25 and 100 μg/ml. Porous TCP granules were used (Peri-OSS™ brand TCP, lot # 7157EL2A2, 40-100 mesh; granules had the size of 150-420 μm and were supplied by DePuy and produced from TCP powder by isostatic pressing and then sintering). The total weight of TCP granules in each dose was 154 mg. The preparations were obtained by aseptically incubating, at 5°C for two hours in a sterile filter unit, TCP particles in either 20 mM sodium acetate buffer, pH 5, or in the two TGF-β1 solutions of the same buffer.

After incubation, TCP granules were harvested by centrifugation to remove the liquid. The bathing solutions were then removed from the particles by microcentrifugation through a filter membrane. Samples that were treated with acetate buffer were labeled as placebo. Samples treated with 100 μg/ml of TGF-β1 were labeled as "high" dose and samples treated with 25 μg/ml of TGF-β1 were labeled as "low" dose. High dose, as indirectly determined by ELISA from the difference in the initial and the final bathing concentration, was 13.7±0.2 μg (± SD, n=3) and low dose was 2.9±0.1 μg (± SD, n=3). The average weight of TCP particles in each vial was 154.1±3.6 mg (± SD, n=8).

The amount of rhTGF-β1 adsorbed onto the TCP granules was determined from the changes in the concentration of TGF-β1 incubating solutions by conventional ELISA methods.

Animal Surgery and Treatment

The animal surgery and treatment were performed as described in Example 4. Sterile TCP or TCP with rhTGF-β1 (3 or 14 μg) was applied to the defect filling the defect. Radiography was performed as described in Example 4, and one hemisection was acid decalcified and one undecalcified as described in Example 4. Goldner's trichrome stained sections were examined using the BioQuant IV™ computer image analysis system as described in Example 4. The thickness of bone at the outside edge of the sections was measured to evaluate bone formation at non-defect sites. In addition, defect area determined radiographically was quantitated using computer image analysis.

Statistical analysis

Statistical analysis was done as described in Example 4. Each group contained two to three rabbits.

Results

Adsorption of TGF-β onto TCP Granules. Figure 3, which shows the adsorption kinetics of TGF-β on TCP granules, indicates that after about 2 hours, the amount adsorbed appears to stabilize, with gradual change up to 22 hours. Figure 4 shows the adsorption of TGF-β on TCP granules, wherein the amount of TGF-β adsorbed is given as a function of bathing concentration of TGF-β. It is seen that the amount of TGF-β adsorbed increases proportionately to the amount of TGF-β in the bathing solution.

Morphometric Determinations. Data from the morphometric determinations are presented in Table 2. In general, TCP with TGF-β1 stimulated a greater degree of bone formation at the defect site compared to TCP without TGF-β1. Indices of bone formation that were increased in defects administered TGF-β1 included osteoid width, % osteoid volume, % osteoid surface, and % osteoblast/total surface. Trabecular bone volume, an indicator of the quantity of bone present within the defect, was significant only at p=0.06. Remodeling of bone was present as indicated by an increase in total resorption surface in the TGF-β1-treated defects compared to placebo-treated defects.

TABLE 2

Histomorphometric Evaluation of Bone Formation in Skull Defects Applied TGF-β1 Impregnated onto 40-100 Mesh TCP Granules[a]			
Histomorphometric Parameters[b]	TGF-β		
	Vehicle	3 μg	14 μg
TBV (%)	1.09±1.09	20.22±5.40[c]	26.18±8.46
OW (mm)	0.002±0.002	0.009±0.001[c]	0.008±0.001[c]
OV (%)	0.03±0.03	9.67±0.75[c]	6.50±2.22[c]
OS (%)	1.39±1.39	33.38±2.66[c]	34.14±9.78[c]
Ob/TS (%)	1.67±1.67	33.42±6.71[c]	38.91±8.28[c]
Ob/Ost (%)	40.00±40.00	98.83±12.20	118.34±7.65
Oc/SL (#)	0.33±0.33	0.57±0.07	0.65±0.24
TRS (%)	0.006±0.006	0.042±0.015[c]	0.041±0.004[c]

[a] Values reported are mean ± S.E.M. based. N = 3 for vehicle, 2 and 3 for 3 or 14 μg of TGF-β1, respectively.
[b] Abbreviations are defined in footnote b of Table 1.
[c] $p < 0.05$.

Radiographic defect area was determined using image analysis techniques. The defect areas were 60.57±9.69, 22.86±7.07, and 8.27±8.27 for placebo, 2, and 14 μg TGF-β1, respectively (Fig. 5). The dose-responsive decrease in defect area was significant for both levels of TGF-β1 ($p < 0.05$). In general, the placebo-treated defects were radiolucent and the TGF-β1-treated defects were radiopaque, except for small centrally located regions in the defects treated with 3 μg TGF-β1.

Histological evaluation. TCP granules (40-100 mesh) with TGF-β1 induced a variable response upon histologic examination. Generally, in defects administered TCP without TGF-β1, the predominant response was a mild chronic inflammation with a mixture of fibrous connective tissue bridging the defect and surrounding the granules of TCP. Minimal bone growth from the margins of the defect were observed in the control group. Defects administered TGF-β1 in TCP induced a much greater bone response with complete bridging in some cases. However, there was mild fibroplasia located within the central portion of the defect and surrounding TCP granules from defects administered 3 μg of TGF-β1. Sometimes the bone formed over or around the granular area with the granules surrounded primarily by fibrous connective tissue. A similar response was observed in defects treated with 14 μg of TGF-β1, with less fibroplasia and more bone formation especially around the granules of TCP.

In summary, radiographs of defect sites after 28 days indicated complete detect closure with 14 μg rhTGF-β1 in 150 mg TCP, having induced a marked increase in bone both surrounding the dorsal and ventral region of TCP granules as well as migrating into the granules. The new bone formed within the defects was characterized histologically as a mixture of immature and mature bone. This indicates active formation and resorption processes that are natural to bone healing. Remodeling of bone was confirmed histomorphometrically by an increase in both bone formation and resorption parameters within TGF-β1-treated sites. The results indicate that the defect area is much lower after application of the TCP granules with 25 μg/ml (3 μg/wound site) TGF-β and is even still lower after application of the TCP granules with 100 μg/ml (14 μg/wound site) TGF-β. TCP granules without TGF-β1 were minimally inductive at 28 days with only slight amounts of bone located at the margins of the defect.

These data show that TGF-β in association with TCP without other carriers such as gelatin functions as a potent bone inducing growth factor, providing a matrix on which bone can readily form across osseous defects.

EXAMPLE 6

Introduction

The purpose of this study was to evaluate the effects of TGF-β1 in the rabbit skull defect model of bone formation when incorporated into TCP granules (150-420 μm) with 12% gelatin that was configured as a disc approximating the size of the defect (12 mm). This was accomplished by measuring selected bone morphometric parameters from stained histologic sections as well as by radiographic examination of the excised defect site. Results were compared to defects

administered TCP in gelatin without TGF-β1.

Source and Preparation of TGF-β and TCP Granules with Gelatin

rhTGF-β1 was prepared as described in Example 1. Individual samples of the active portion of rhTGF-β1 were prepared under sterile conditions in 20 mM sodium acetate buffer at pH 5.0.

The solution of rhTGF-β1 in 12% gelatin was prepared by dissolving gelatin (type A, 300 Bloom grams) in 20 mM sodium acetate, pH 5.0 with moderate heat. The gel solution was sterilized by membrane filtration while it was still very warm. As the solution was cooled to a temperature below 50°C, an appropriate aliquot of the sterile rhTGF-β1 solution was added and homogeneously mixed. After mixing, 400 µl of this gelatin-TGF-β1 solution was pipetted into 5-ml vials that contained 300 mg of TCP granules (150-420 µm, DePuy). The preparation was allowed to congeal. By varying the added volume of TGF-β1 solution, the final doses were 0, 5, and 21.5 µg TGF-β1 per disc of TCP-gelatin.

Animal Surgery and Treatment

The animal surgery and treatment were performed as described in Example 4. Sterile TCP/gelatin or TCP/gelatin with TGF-β1 (5 or 21.5 µg) was applied to the defect filling the defect. Radiography was performed as described in Example 4, and one hemisection was acid decalcified and one undecalcified as described in Example 4. Goldner's trichrome stained sections were examined using the BioQuant IV™ computer image analysis system as described in Example 4. In addition, the defect area determined radiographically was quantitated using computer image analysis.

Statistical analysis

Statistical analysis was done as described in Example 4. Each group contained 5 to 6 rabbits.

Results

Morphometric Determinations. Data from the morphometric determinations are presented in Table 3. In general, TGF-β1 formulated in TCP and 12% gelatin stimulated a much greater degree of bone formation at the defect site compared to TCP in 12% gelatin without TGF-β1. All indices of bone formation were increased in defects administered either 5 or 21.5 µg TGF-β1. The number of osteoclasts/surface length and total resorption surface were increased in defects treated with 5 µg, but not 21.5 µg, TGF-β1, indicating that remodeling processes were present at least for the lower dose of the growth factor.

TABLE 3

Histomorphometric Evaluation of Bone Formation in Skull Defects Applied TGF-β1 in 300 mg TCP/12% Gelatin[a]			
Histomorphometric Parameters[b]	TGF-β		
	Vehicle	5 µg	21.5 µg
TBV (%)	0	14.11±2.12[c]	20.20±4.09[c]
OW (mm)	0	0.01±.001[c]	0.01±0.001[c]
OV (%)	0	7.13±0.99[c]	8.34±1.52[c]
OS (%)	0	41.15±3.01[c]	50.38±2.84[c]
Ob/TS (%)	0	43.34±6.28[c]	57.34±3.86[c]
Ob/Ost (%)	0	104.37±14.04[c]	114.17±5.34[c]
Oc/SL (#)	0	0.47±0.11[d]	0.23±0.11
TRS (%)	0	0.029±0.009[d]	0.012±0.006

[a] Values reported are mean ± S.E.M. N = 5 for vehicle, and 6 each for 5 or 21.5 µg of TGF-β1.
[b] Abbreviations are defined in footnote b of Table 1.
[c] $p < 0.01$.
[d] $p < 0.05$.

Radiographic defect area was determined using image analysis techniques. The defect areas were 67.49 ± 6.57, 37.89 ± 5.14, and 23.24 ± 7.99 for placebo, 5, and 21.5 μg of TGF-β1, respectively (Fig. 6). The dose-responsive decrease in defect area was significant for both levels of TGF-β1 ($p < 0.01$). Radiographs were difficult to interpret morphometrically since the granules of TCP were radiopaque. However, the general appearance of the TGF-β1-treated defects was denser, especially within the center of the defect.

Histologic Evaluation. TCP in 12% gelatin with TGF-β1 induced a variable response upon histologic examination. Generally, the predominant response was a mild chronic inflammation with a mixture of fibrous connective tissue bridging the defect and surrounding the granules of TCP in the defect administered TCP in 12% gelatin without TGF-β1. Minimal bone growth from the margins of the defect were observed in the placebo group. Defects administered TGF-β1 in TCP and 12% gelatin induced a much greater bone response with complete bridging in most defects administered 5 μg of TGF-β1. However, there was a mild chronic inflammatory response with fibroplasia located within the central portion of the defect and surrounding TCP granules from defects administered 5 μg of TGF-β1. Four of five defects administered 21.5 μg of TGF-β1 were completely bridged with bone. However, mild chronic inflammation in this group was still evident at each site with variable amounts of fibrous connective tissue intermingled with granules of TCP.

In summary, the TCP granules in 12% gelatin with TGF-β1 induced a marked increase in bone both surrounding the space occupied by the granules as well as interspersed in the granules. Bone was characterized histologically as a mixture of immature and mature bone, indicating active formation and resorption processes. Remodeling of bone was subsequently confirmed histomorphometrically by an increase in both formation and resorption parameters within TGF-β1-treated sites. When compared to the TGF-β1-impregnated TCP disc study, however, the values from histomorphometry were lower in the TCP granule/12% gelatin formulation, indicating that the bone response was not as vigorous. Also, the formulation melted rapidly and was not easily conformable to the defect.

These data demonstrate with the other examples that TCP will function as a carrier for TGF-β1 and provide a matrix on which bone can readily form across osseous defects. It is believed that the mild chronic inflammation would resolve with time as bone replaced the granules of TCP.

The same experiment is expected to yield similar results using a gelatin/agarose mixture containing, for example, about 0.05-1 % (weight/weight) agarose, with an exemplary amount being 0.25%, to increase the melting point of the composition.

EXAMPLE 7

Introduction

The purpose of this study was to evaluate the effects of TGF-β1 in the rabbit skull defect model of bone formation when incorporated into TCP granules with 2% lyophilized gelatin that was configured as a disc of material approximating the size of the defect (12 mm). This was accomplished by measuring selected bone morphometric parameters from stained histologic sections as well as by radiographic examination of the excised defect site. Results were compared to defects administered large granules of TCP in lyophilized gelatin without TGF-β1.

Source and Preparation of TGF-β and TCP Particles and Gelatin

rhTGF-β1 was prepared as described in Example 1 and formulated in TCP with 2% gelatin as follows. A solution of 2% gelatin (type A, 300 Bloom grams) with 2% glycerol was prepared in 20 mM sodium acetate, pH 5.0 and sterilized by filtration. An aliquot amount of sterile TGF-β1 solution (20 or 50 μg) was added into the gelatin mixture at a temperature of about 50°C and homogeneously mixed at that temperature to form a gel solution. TCP particles (500 mg, sized at 420-2000 μm) were weighed into sterile siliconized vials. The gel solution (0.5 ml) was then added onto the TCP granules, sufficiently to cover all the granules. The preparation was subsequently lyophilized by conventional lyophilization technology. The final doses in these preparations were 20 and 50 μg.

Animal Surgery and Treatment

The animal surgery and treatment were performed as described in Example 4. Sterile large granules of TCP in lyophilized 2% gelatin without TGF-β (placebo) or with TGF-β1 (20 or 50 μg) were applied to the defect filling the defect. Radiography was performed and one hemisection was acid decalcified and one undecalcified as described in Example 4. Goldner's trichrome stained sections were examined using the BioQuant IV™ computer image analysis system as described in Example 4. The thickness of bone at the outside edge of the sections was measured to evaluate bone formation at non-defect sites. In addition, defect area determined radiographically was quantitated using computer image analysis.

Statistical analysis

Statistical analysis was done as described in Example 4. Each group contained four to five rabbits.

Results

Morphometric Determinations. Data from the morphometric determinations are presented in Table 4. In general, the baseline values for the placebo control group were relatively high, indicating that large granules of TCP in lyophilized gelatin induced bone formation to a greater extent than other formulations. However, 20 μg TGF-β1 stimulated more bone formation at the defect site compared to TCP in gelatin without TGF-β1 as indicated by an increase in trabecular bone volume and % osteoblast/total surface. In contrast, 50 μg TGF-β1 did not induce an increase in bone formation compared to the TCP placebo except for % osteoblast total surface. Non defect end width was similar between groups.

TABLE 4

Histomorphometric Evaluation of Bone Formation in Skull Defects Applied TGF-β1 with Large Granules of TCP in Lyophilized Gelatin[a]			
Histomorphometric Parameters[b]	TGF-β		
	Vehicle	20 μg	50 μg
TBV (%)	15.66±1.74	28.93±2.65[c]	15.43±2.58
OW (mm)	0.011±0.003	0.009±0.001	0.009±0.001
OV (%)	5.04±0.58	7.31±2.10	8.25±1.23
OS (%)	30.27±3.89	41.42±6.68	40.68±4.25
Ob/TS (%)	35.95±2.24	50.47±3.98[d]	54.12±4.84[d]
Ob/Ost (%)	123.99±11.03	129.62±17.65	140.54±22.04
Oc/SL(#)	0.32±0.39	0.56±0.16	0.33±0.18
TRS (%)	0.015±0.003	0.029±0.009	0.017±0.007
NDEW (mm)	1.55±0.11	1.80±0.12	1.90±0.07

[a] Values reported are mean ± S.E.M. based. N = 5 for vehicle, 4 and 5 for 20 and 50 μg of TGF-β1, respectively.
[b] Abbreviations are defined in footnote b of Table 1.
[c] $p < 0.01$.
[d] $p < 0.05$.

Radiographic defect area was determined using image analysis techniques. The defect areas were 27.22±7.84, 4.86±2.25, and 25.01±7.06 for placebo, 20, and 50 μg TGF-β1, respectively (Fig. 7). The decrease in defect area was significant for 20 μg of TGF-β1 only ($p < 0.01$). Radiographs were difficult to interpret morphometrically, since the large granules of TCP were radiopaque and unevenly distributed over the defect area. However, the general appearance of the defects administered 20 μg of TGF-β1 was denser and filled the defect.

Histologic Evaluation. Histologic examination of bone samples from defects administered TCP in lyophilized gelatin without TGF-β1 indicates bone formation at the margin of the defect bridging approximately 50% of the cross section. Where bone was present there appeared to be trabeculae of bone lined by osteoblasts surrounding the large granules of TCP. Marrow cavities were present with a typical cellular pattern for bone. In the central 50% of the defect from placebo-treated defects the large granules were surrounded by fibroblasts and fibrous connective tissue. Defects administered 20 μg TGF-β1 in large granules of TCP and lyophilized gelatin induced a much greater bone response with complete bridging in all cases. However, there were small areas of fibroplasia occasionally located within the central portion of the defect and surrounding TCP granules. Histologic examination indicated that defects administered 50 μg TGF-β1 induced a variable response. Two of five defects administered 50 μg TGF-β1 were completely bridged with bone, while 2 of 5 defects contained predominantly a fibrous response with minimal bone from the margins of the defect. In each case at 50 μg TGF-β1, there was a thick layer of fibrous connective tissue over the periosteal surface.

In summary, the large TCP granules in lyophilized gelatin with 20 μg TGF-β1 induced a moderate increase in bone both surrounding the space occupied by the granules as well as interspersed in the granules. Bone was characterized

histologically as a mixture of immature and mature bone, indicating active formation and resorption processes. When compared to other formulations of TCP without TGF-β1, there was a substantial increase in the baseline amount of bone in the TCP placebo group. Without being limited to any one theory, this effect could be attributed to the size of the TCP granules, which are known to be conductive, as well as to the lyophilized gelatin formulation. The low dose of TGF-β1 (20 μg) induced an increase in bone compared to both the TCP placebo and 50 μg TGF-β1. While morphometrically there were fewer parameters that were significantly different from TCP placebo than in the other TCP studies, the low dose of TGF-β1 appeared very comparable to similar doses of TGF-β1 in other formulations. In contrast, 50 μg TGF-β1 was remarkably different, with a much greater degree of fibroplasia and a much more variable amount of bone. This indicates that in this model there is a biphasic response with TGF-β1 similar to other models of soft tissue wound healing.

These data further demonstrate that TCP will function as a carrier for TGF-β and provide a matrix on which bone can readily form across osseous defects.

EXAMPLE 8

The purpose of this study was to evaluate the effects of TGF-β1 in the rabbit skull defect model of bone formation when incorporated into TCP granules (5 μm or 250 μm nominal particle size) with amylopectin that was configured as a malleable putty approximating the size of the defect (12 mm). In addition, the individual components, i.e., TCP (5 or 250 μm) and two different lots of amylopectin were evaluated to determine which component contributed to the incidence of giant cell formation observed in this model. Defect sites were removed 28 days after surgery, radiographed, and processed for histomorphometric determinations. Introduction

In the rabbit skull defect model, there appears to be a foreign body giant cell response. The purpose of this study was to evaluate in this model the effects of the individual components of the formulation and combinations of TGF-β1 and two sizes of TCP granules (nominal 5 or 250 μm granules) formulated in two lots of amylopectin having different levels of endotoxin present. Histologic examination with measurement of selected bone morphometric parameters from stained histologic sections as well as radiographic examination of the excised defect site were used as criteria for efficacy.

Source and Preparation of TGF-β1 and TCP/amylopectin

Types of Formulations tested. Eight groups of formulations were evaluated for efficacy in the animal model: two amylopectin controls, two vehicle controls, and the TGF-β1-treated groups as described below:

Group 1: Amylopectin with 12 EU/g
Group 2: Amylopectin with > 3500 EU/g
Group 3: 5 μm TCP and amylopectin
Group 4: Amylopectin and 250 μm TCP
Group 5: Amylopectin and 10 μg TGF-β1
Group 6: Amylopectin and 5 μm TCP and 10 μg TGF-β1
Group 7: Amylopectin and 250 μm TCP and 10 μg TGF-β1
Group 8: 10 μg TGF-β1 and 250 μm TCP and amylopectin*

Preparation of Formulations. rhTGF-β1 was prepared as described in Example 1. Individual samples of the active portion of the TGF-β1 were prepared under sterile conditions in 20 mM sodium acetate buffer at pH 5.0. Two different ranges of particle size of TCP were used to prepare the paste, 5 and 250 μm (nominal, range = 5 - 45 μm and 250-500 μm, respectively). Aseptic conditions were maintained throughout the preparation procedure. The TCP granules were sterilized by 2.5 MRAD gamma irradiation.

Two lots of amylopectin (potato, Sigma Chemical Co.) with different levels of endotoxin (12 EU/g and >3500 EU/g) were used in Groups 1 and 2, respectively. Only the 12-EU/g amylopectin was used in Groups 3-8.

The TCP/amylopectin paste for Group 7 was prepared by mixing sterile TCP granules and sterile amylopectin in the ratio of 4:1 and 2:1 (by weight) for TCP granules with particle size of <45 and 250-500 μm, respectively. An aliquot of TGF-β1 solution in 20 mM acetate buffer, pH 5, was added to the solid mixture. The mixing was then performed manually using a spatula and plate until a uniform mass was obtained. In each preparation, the volume of TGF-β1 solution was kept constant at the ratio of 1:0.4 (weight of TCP:volume of TGF-β1 solution). The amount of amylopectin/TCP paste administered into each animal was about 500 mg, with the final dose of 10 μg TGF-β1.

For Group 8, the TGF-β solution was mixed with the TCP sufficiently to become adsorbed thereon, and then the amylopectin was mixed in to homogeneity.

* Group 8 differs from group 7 only in the order of mixing.

Animal Surgery and Treatment

The animal surgery and treatment were performed as described in Example 4 using the eight groups of formulations defined above. The formulations were malleable, having the consistency of putty, and were applied to the defect filling the space completely. Radiography was performed as described in Example 4, and one hemisection was acid decalcified and one undecalcified as described in Example 4.

The decalcified and undecalcified stained sections were evaluated for general characteristics and quality of healing, especially for the presence or absence of a foreign body giant cell response. In addition, Goldner's trichrome stained sections were examined using the BioQuant IV™ computer image analysis system as described in Example 4. In addition, defect area determined radiographically was quantitated using computer image analysis.

Statistical analysis

Statistical analysis was done as described above. Each group contained two to three rabbits.

Results

Histologic Evaluation. A summary of the histopathologic evaluation is presented in Table 5. Both lots of amylopectin induced a minimal bone response for foreign body giant cell response. In contrast, the amylopectin with 5 μm or 250 μm TCP granules induced a mixed response with minimal bone formation and a marked foreign body giant cell response. Defect sites administered amylopectin with 10 μg TGF-β1 but without TCP exhibited extensive new bone formation with minimal foreign body giant cell response. Ten μg TGF-β1 administered to defects with amylopectin and 5 μm TCP induced a variable response with new bone formation as well as a moderate giant cell response. When 10 μg TGF-β1 was mixed with 250 μm TCP and then mixed with amylopectin, the amount of bone formation was increased to a level similar to the growth factor plus amylopectin formulation and the degree of giant cell formation was minimal to moderate. In contrast, when the mixing order was reversed such that the TCP and amylopectin were mixed first, the TGF-β1 added less bone and a greater degree of giant cell formation occurred.

TABLE 5

Summary of Histologic Evaluation of Hematoxylin- and Eosin-Stained Sections	
Group	
1	Minimal bone response; connective tissue bridge; minimal giant cell response.
2	Minimal bone response; connective tissue bridge; minimal giant cell response.
3	Minimal to no bone response; very reactive, with numerous giant cells throughout.
4	Minimal to no bone response; moderate fibrosis; numerous giant cells surrounding large cavities (presumably decalcified TCP).
5	Complete bridging of defect with bone; profound increase in osteoblasts; minimal signs of chronic inflammation; thick fibrotic capsule overlying bone.
6	75-90% bridging of defect with bone; central area contains moderate fibrosis; moderate giant cell response with lots of debris (small particles); bone looks good where it is present; in one sample the bone appears to be primarily periosteal with gaps at the original cut edges.
7	0-90% bridging of defect with bone; 1/3 - severe giant cell response; small amount of connective tissue; 1/3 - center area moderate giant cell response with chronic inflammation; 1/3 - new bone looks good, funnels down centrally with mild giant cell response with debris.
8	75-100% bridging of defect with bone; minimal (2/3) to moderate (1/3) giant cell response; thick fibrous response overlying bone; predominant response is one of large amounts of bone surrounding small cavities (decalcified TCP).

Morphometric Determinations. Data from the morphometric determinations are presented in Table 6 and Figure 8. No measurements could be determined in the vehicle groups that contained either lot of amylopectin. In general, TGF-β1 formulated with 250 μm TCP, then mixed with amylopectin, stimulated a much greater degree of bone formation at the defect site compared to the other formulations except for the TGF-β1 and amylopectin combination. Most osteoblastic and osteoclastic indices were increased in the TGF-β1-treated groups compared to groups without TGF-β1. Radiographic defect area was determined using image analysis techniques and is illustrated in Figure 9. The differences

between groups typically depended on the presence or absence of TGF-β1. The defect area tended to be smaller for the non-TGF-β1-treated groups that contained TCP granules that were 250 μm. However, radiographs were difficult to interpret morphometrically due to the radiopacity of the TCP granules in all but the first two groups, i.e., the two lots of amylopectin alone.

TABLE 6

Histomorphometric Evaluation of Bone Formation in Skull Defects Histomorphometric[a] Parameters							
Group	Trabecular Bone Vol. (%)	Osteoid Width (mm)	Osteoid Volume (%)	Osteoid Surface (%)	Osteoblast/Total Surface (%)	Osteoblast/Osteoid (%)	Osteoclast/Surface length (#)
1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3	2.68 (2.68)	0.003 (0.003)	1.10 (1.10)	5.41 (5.41)	7.54 (7.54)	46.40 (46.40)	0.10 (0.10)
4	2.27 (2.27)	0 (0)	0 (0)	0 (0)	25.01 (25.01)	0 (0)	0 (0)
5	31.27 (1.28)	0.01 (0)	7.63 (1.42)	49.56 (6.49)	51.56 (9.26)	102.51 (5.83)	0.54 (0.27)
6	16.71 (1.99)	0.012 (0.004)	12.06 (5.15)	38.38 (11.39)	52.90 (6.85)	166.75 (48.83)	0.46 (0.11)
7	16.82 (8.54)	0.014 (0.009)	8.21 (6.04)	22.41 (11.51)	28.59 (14.30)	86.9 (44.97)	0.14 (0.07)
8	30.78 (6.26)	0.016 (0.002)	13.20 (3.53)	51.29 (7.02)	45.33 (4.88)	89.55 (6.73)	0.40 (0.21)

[a] Data are expressed as mean (S.E.M.).

In summary, results from this study indicate that the amount of endotoxin present in the amylopectin did not affect the amount of giant cell formation and therefore indicates that amylopectin should be an adequate carrier for TCP and TGF-β1. In contrast, both the 5 μm and 250 μm TCP induced giant cell formation when mixed with the low-endotoxin amylopectin. It was determined retrospectively that the 250 μm TCP contained TCP powder (particles < 45 μm). Since the degree of giant cell formation was less in the 250 μm TCP than the 5 μm TCP, without being limited to any one theory, it is believed that the small TCP granules in the 250 μm TCP formulation may be contributing to the level of giant cell formation.

The defect area measured from radiographs was similar between groups administered amylopectin alone or 5 μm TCP granules alone. The defect areas for sites administered amylopectin and 250 μm TCP granules (with or without TGF-β1) were similar and all were smaller than sites administered 5 μm TCP granules alone or amylopectin alone.

Morphometric parameters were similar among the TGF-β-treated groups. Histopathologic examination of the eight microscopic slides indicates that the overall response of the TGF-β1 formulated in 250 μm TCP was better than that of the TGF-β1 formulated in 5 μm TCP. A moderate to severe giant cell foreign body reaction was observed with the 5 μm TCP in amylopectin with or without TGF-β1.

These data indicate that TCP/amylopectin will function as a carrier for TGF-β and provide a matrix on which bone can readily form across osseous defects.

EXAMPLE 9

The purpose of this study was to evaluate the effects of TGF-β1 in the rabbit long-bone model of bone formation when incorporated into TCP granules (5 μm or 250 μm nominal particle size) with amylopectin that was configured as a malleable putty the approximate size of the defect. Defect sites were radiographed and processed for histomorphometric determinations. Source and Preparation of TGF-β1 and TCP/amylopectin

rhTGF-β1 was prepared as described in Example 1. Individual samples of the active portion of the rhTGF-β1 were prepared under sterile conditions in 20 mM sodium acetate buffer at pH 5.0. A 4% solution of amylopectin (potato, Sigma Chemical Co.) was prepared by adding amylopectin to water and sterilizing in an autoclave at 100-120°C for no less than 30 minutes. The solution was filtered through a 0.22-μm membrane. For removal of all water, the sterile amylopectin solution was lyophilized.

The sterile TGF-β1 solution was adsorbed onto the TCP granules (125-250 μm) by aseptic incubation in a sterile filter unit at 5°C for 2 hours as described in Example 5. The amylopectin was aseptically mixed with the TCP granules upon which the TGF-β1 solution was adsorbed using plate and spatula to homogeneity.

The proportions of amylopectin, TCP, and volume of water from the TGF-β1 solution were varied according to the particle size of TCP. In this study, three ranges of particle size were used, < 5 μm, ≥ 75 μm, and ≥ 125 μm. The propor-

tion (by weight) of TCP:amylopectin was 1:0.25 when the TCP particles were < 5 μm. The percentage of water needed in the mixing was 30% (volume/weight of the total amount of solids). For TCP with larger particle sizes (≥ 75 μm and ≥ 125 μm), the ratio of TCP:amylopectin:TGF-β solution (weight/weight) was 1:0.5:0.5.

Release of TGF-β from TCP/Amylopectin Formulation

Figure 10 shows a graph of the percent of TGF-β released over time from the amylopectin/TCP formulation of 250 to 400 μm particle size into normal human serum (open circles) and into phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) (solid circles). The release of the TGF-β was measured by standard ELISA methods. It can be seen that the TGF-β is released much more quickly from the TCP/amylopectin in normal human serum than in the PBS.

Animal Surgery and Treatment

A model of repair of long bone discontinuities in rabbits was devised based on that provided in Lemons et al., *supra*. All studies were performed in accordance with the AAALAC guidelines. Male New Zealand White rabbits (2.8-3.2 kg) (Elkhorn Rabbitry, Watsonville, CA) were anesthetized with 0.75 ml/kg Hypnorm® brand anesthesia (Jenssen Pharmaceutica, Beersa, Belgium). The right forelimb from each rabbit was shaved and aseptically prepared for surgery. An incision was made over the anterior-medial aspect of the forearm (radium/ulna), reflecting the skin laterally. The muscles surrounding the radius were bluntly reflected from the field of view and about 1.5 cm of the radius was exposed. A 10-mm section of the mid-shaft radius was removed using an electric drill while liberally irrigating with physiological saline during the drilling to prevent overheating of the bone margins. Care was taken not to damage the adjacent ulna. After the 10-mm section of bone was removed, the gap was packed with sterile gauze to facilitate hemostasis. The defect site was subsequently irrigated to eliminate any small particles of bone.

Three groups were evaluated in the initial preliminary investigations. The vehicle control group was treated with a formulation consisting of sterile TCP (125 μm particle size) mixed with amylopectin to homogeneity. The TGF-β1-treated group was treated with a mixture of TCP, amylopectin, and 15 μg TGF-β1 formulated as described above. The third treatment group consisted of a 10-mm defect without any treatment. The formulation with TGF-β1, amylopectin, and TCP was malleable, having the consistency of putty, and was applied to the defect filling the space completely. The reflected muscles were sutured back in place and the skin was closed with 4-0 silk. Rabbits were returned to their cages and allowed to recover.

Immediately after surgery and weekly thereafter, the surgical site from each rabbit was radiographed to monitor healing. After 28 days rabbits were euthanized with an overdose of barbiturate and the radius and ulna were removed and excess soft tissue (i.e., muscle) was dissected away from the bone and defect site. Sites were fixed in 10% neutral buffered formalin. The fixed tissue samples were then acid decalcified (using Easy-cut™ reagent. American Histology Reagent Co., Modesto, CA), and serial sections were processed by routine histologic methods using hematoxylin and eosin to stain the 4-μm sections.

The decalcified stained sections were evaluated for general characteristics and quality of healing. In addition, representative longitudinal sections taken from the center of the defect were examined using a BioQuant IV™ computer image analysis system. Selected indices of bone formation and resorption were measured including TBV, %OS, %OV, OW, %Ob/Ost, %Ob/TS, TRS, and #Oc/SL. Sections from all animals were analyzed histomorphometrically using a random stratified sampling scheme that systematically evaluated selected fields from the bony edge of the defect and the entire area within the defect. Fields were selected using a grid pattern, such that each field within the defect area had an equal probability of being selected. Approximately equal numbers of fields were evaluated for both the control and treated defects.

Statistical analysis

Statistical analysis was done as described above. Each group contained 3 to 4 rabbits.

Results

Preliminary results from radiographs indicate that the defect filled more rapidly in rabbits administered TGF-β1 formulated with TCP plus amylopectin than the untreated control or the groups with TCP plus amylopectin alone. The defects administered TGF-β1 tended to be filled with radiodense material by 21 days, while defects administered TCP plus amylopectin alone were less dense radiographically at 21 or 28 days. Defects that were untreated exhibited minimal filling within the 28-day observation period. Histologic data is expected to confirm the radiographic data, in that the TGF-β1 /TCP/amylopectin formulation is expected to increase most if not all histomorphometric parameters examined to a greater extent than the other two control formulations.

The result of wetting the TCP with the TGF-β first before adding amylopectin rather than adding the TGF-β to the mixture of TCP and amylopectin was a better pharmacological effect. Without being limited to any one theory, it is believed that the better efficacy of the preparation wherein TGF-β is first adsorbed onto TCP is due to the ability of the osteoblasts to form around the TCP particles where the TGF-β was localized.

These data further indicate that TCP/amylopectin will function as a carrier for TGF-β and provide a matrix on which bone can readily form across osseous defects. The TCP/amylopectin formulation is preferred in that it does not melt as rapidly as those with gelatin and could evenly disperse the large TCP granules yet be malleable and formable to regular defects like a putty.

EXAMPLE 10

The purpose of this study was to formulate the TGF-β in collagen and TCP.

Collagen CN (Prodex, Inc., Princeton, NJ) was sterilized by ethylene oxide. The matrix was prepared by mixing an appropriate aliquot of rhTGF-β1 solution prepared as described in Example 4 with TCP (≤ 5 μm) and collagen aseptically using plate and spatula. The proportion of TCP:collagen:water was 6:1:6 (weight: weight: volume). The volume of water needed was replaced by the sterile TGF-β1 solution. The final dose that can be administered for the rabbit skull defect model is about 8-10 μg of TGF-β1, depending on the defect size. The amount of TCP powder in each studied animal can be about 500-750 mg.

The radiographic data indicated that the formulation of collagen + TCP + 10 μg TGF-β was significantly ($p < 0.05$) more efficacious than collagen alone in the rabbit skull defect model described above.

Claims

1. A bone-inducing formulation comprising an effective amount of transforming growth factor-β and tricalcium phosphate, wherein the tricalcium phosphate is in the form of particles on which the transforming growth factor-β is adsorbed, the formulation excluding activin and bone morphogenetic cofactor.

2. The formulation of claim 1 wherein the particles are granules or a powder.

3. The formulation of claim 2 wherein the tricalcium phosphate is in the form of granules with a diameter of about 120 to 500 μm.

4. The formulation of any one of the preceding claims further comprising an effective amount of a polymer for enhancing consistency of the formulation.

5. The formulation of claim 4 wherein the polymer is amylopectin, gelatin, collagen, agarose, or a mixture of two or more of these polymers.

6. The formulation of claim 5 wherein the polymer is lyophilized before use.

7. A bone-inducing formulation comprising about 0.5 μg to about 5 mg transforming growth factor-β adsorbed onto about 140 mg to about 50 g of tricalcium phosphate particles, the formulation excluding activin and bone morphogenetic cofactor.

8. The formulation of claim 7 wherein about 1 μg to about 3 mg transforming growth factor-β is adsorbed onto the tricalcium phosphate particles.

9. The formulation of claim 7 wherein the size of the particles is about 120-500 μm.

10. The formulation of claim 7 wherein the size of the particles is about 125-250 μm.

11. A bone-inducing formulation comprising about 0.5 μg to about 5 mg transforming growth factor-β, about 140 mg to about 50 g tricalcium phosphate particles, and an amount of amylopectin that ranges from about 0.1:1 to 1:1 amylopectin:tricalcium phosphate.

12. The formulation of claim 11 wherein the amount of amylopectin ranges from about 0.25:1 to 0.5:1 amylopectin:tricalcium phosphate.

13. The formulation of claim 11 wherein the size of the particles is no less than about 75 μm and the ratio of tricalcium

phosphate:amylopectin:TGF-β solution is about 1:0.5:0.5.

14. The formulation of claim 11 wherein the size of the particles is about 120-500 μm.

15. A method of producing a bone-inducing formulation of transforming growth factor-β comprising admixing an effective amount of a liquid solution of the transforming growth factor-β with tricalcium phosphate granules for a sufficient period of time to adsorb the transforming growth factor-β onto the granules and contacting the resulting mixture with an effective amount of amylopectin.

16. The method of claim 15 wherein the size of the granules is greater than about 100 μm.

Patentansprüche

1. Formulierung zur Induktion von Knochenwachstum, umfassend eine wirksame Menge an Transformationswachstumsfaktor-β und Tricalciumphosphat, worin das Tricalciumphosphat die Form von Teilchen aufweist, auf denen der Transformationswachstumsfaktor-β adsorbiert ist, wobei die Formulierung kein Activin und keinen knochenmorphogenetischen Cofaktor enthält.

2. Formulierung nach Anspruch 1, worin die Teilchen Granulat oder Pulver sind.

3. Formulierung nach Anspruch 2, worin das Tricalciumphosphat in Form von Körnchen mit einem Durchmesser von etwa 120 bis 500 μm vorliegt.

4. Formulierung nach einem der vorhergehenden Ansprüche, weiters umfassend eine wirksame Menge eines Polymers, um die Konsistenz der Formulierung zu verbessern.

5. Formulierung nach Anspruch 4, worin das Polymer Amylopectin, Gelatine, Collagen, Agarose oder ein Gemisch zweier oder mehrerer dieser Polymere ist.

6. Formulierung nach Anspruch 5, worin das Polymer vor der Verwendung lyophilisiert wird.

7. Formulierung zur Induktion von Knochenwachstum, umfassend etwa 0,5 μg bis etwa 5 mg Transformationswachstumsfaktor-β, der auf etwa 140 mg bis etwa 50 g Tricalciumphosphatteilchen adsorbiert ist, wobei die Formulierung kein Aktivin und keinen knochenmorphogenetischen Cofaktor enthält.

8. Formulierung nach Anspruch 7, worin etwa 1 μg bis etwa 3 mg Transformationswachstumsfaktor-β auf den Tricalciumphosphatteilchen adsorbiert sind.

9. Formulierung nach Anspruch 7, worin die Größe der Teilchen etwa 120-500 μm beträgt.

10. Formulierung nach Anspruch 7, worin die Größe der Teilchen etwa 125-250 μm beträgt.

11. Formulierung zur Induktion von Knochenwachstum, umfassend etwa 0,5 μg bis etwa 5 mg Transformationswachstumsfaktor-β, etwa 140 mg bis etwa 50 g Tricalciumphosphatteilchen und eine Menge an Amylopectin, die von etwa 0,1:1 bis 1:1 Amylopectin:Tricalciumphosphat reicht.

12. Formulierung nach Anspruch 11, worin die Menge an Amylopectin von etwa 0,25:1 bis 0,5:1 Amylopectin:Tricalciumphosphat reicht.

13. Formulierung nach Anspruch 11, worin die Größe der Teilchen zumindest etwa 75 μm beträgt und das Verhältnis von Tricalciumphosphat:Amylopectin:TGF-β-Lösung etwa 1:0,5:0,5 ist.

14. Formulierung nach Anspruch 11, worin die Größe der Teilchen etwa 120-500 μm beträgt.

15. Verfahren zur Herstellung einer Formulierung des Transformationswachstumsfaktors-β zur Induktion von Knochenwachstum, umfassend das Vermischen einer wirksamen Menge einer flüssigen Lösung des Transformationswachstumsfaktors-β mit Tricalciumphosphatgranulat über einen Zeitraum, der ausreicht, um den Transformationswachstumsfaktor-β am Granulat zu adsorbieren, und das In-Kontakt-Bringen des resultierenden Gemisches mit einer wirksamen Menge an Amylopectin.

16. Verfahren nach Anspruch 15, worin die Größe der Granulatteilchen größer als etwa 100 µm ist.

Revendications

1. Formulation induisant l'ostéogenèse comprenant une quantité efficace de facteur de croissance transformant β et de phosphate tricalcique, dans laquelle le phosphate tricalcique est sous forme de particules sur lesquelles se trouve adsorbé le facteur de croissance transformant β, la formulation excluant l'activine et le cofacteur morphogénétique de l'os.

2. Formulation selon la revendication 1, dans laquelle les particules sont des granules ou une poudre.

3. Formulation selon la revendication 2, dans laquelle le phosphate tricalcique est sous forme de granules ayant un diamètre d'environ 120 à 500 µm.

4. Formulation selon l'une quelconque des revendications précédentes comprenant en outre une quantité efficace d'un polymère pour améliorer la consistance de la formulation.

5. Formulation selon la revendication 4, dans laquelle le polymère est l'amylopectine, la gélatine, le collagène, la gélose, ou un mélange de deux ou de plusieurs de ces polymères.

6. Formulation selon la revendication 5, dans laquelle le polymère est lyophilisé avant emploi.

7. Formulation induisant l'ostéogenèse comprenant de 0,5 µg environ à 5 mg environ de facteur de croissance transformant β adsorbé sur environ 140 mg à environ 50 g de particules de phosphate tricalcique, la formulation excluant l'activine et le cofacteur morphogénétique de l'os.

8. Formulation selon la revendication 7, dans laquelle environ 1 µg à environ 3 mg de facteur de croissance transformant β sont adsorbés sur les particules de phosphate tricalcique.

9. Formulation selon la revendication 7, dans laquelle la taille des particules s'échelonne d'environ 120 à 500 µm.

10. Formulation selon la revendication 7, dans laquelle la taille des particules s'échelonne d'environ 125 à 250 µm.

11. Formulation induisant l'ostéogenèse comprenant de 0,5 µg environ à 5 mg environ de facteur de croissance transformant β, de 140 mg environ à 50 g environ de particules de phosphate tricalcique, et une quantité d'amylopectine qui s'échelonne d'environ 0,1:1 à 1:1 en termes d'amylopectine:phosphate tricalcique.

12. Formulation selon la revendication 11, dans laquelle la quantité d'amylopectine s'échelonne d'environ 0,25:1 à 0,5:1 en termes d'amylopectine:phosphate tricalcique.

13. Formulation selon la revendication 11, dans laquelle la taille des particules est non inférieure à environ 75 µm et le rapport de la solution phosphate tricalcique:amylopectine:TGF β est d'environ 1:0,5:0,5.

14. Formulation selon la revendication 11, dans laquelle la taille des particules est d'environ 120 à 500 µm.

15. Procédé de production d'une formulation induisant l'ostéogenèse à base de facteur de croissance transformant β comprenant le mélange d'une quantité efficace d'une solution liquide du facteur de croissance transformant β avec des granules de phosphate tricalcique pendant un laps de temps suffisamment long pour adsorber le facteur de croissance transformant β sur les granules et la mise en contact du mélange obtenu avec une quantité efficace d'amylopectine.

16. Procédé selon la revendication 15, dans lequel la taille des granules est supérieure à environ 100 µm.

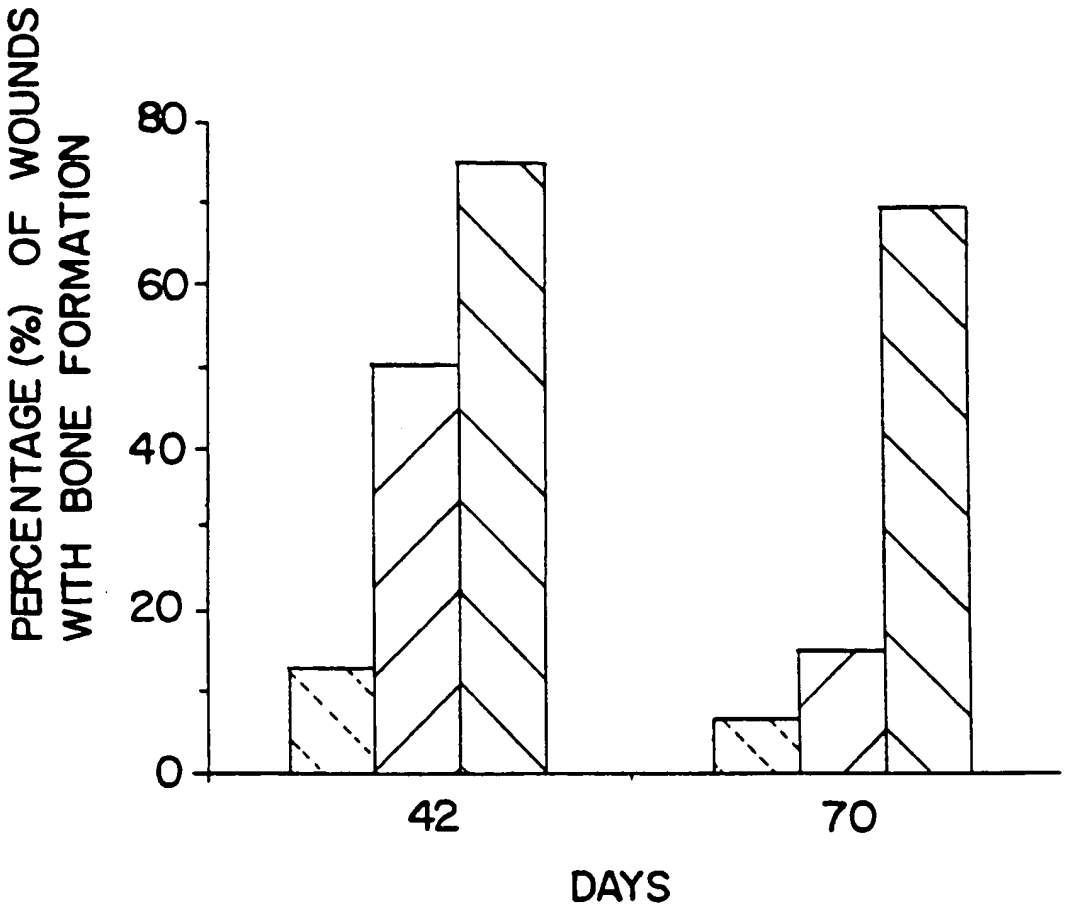
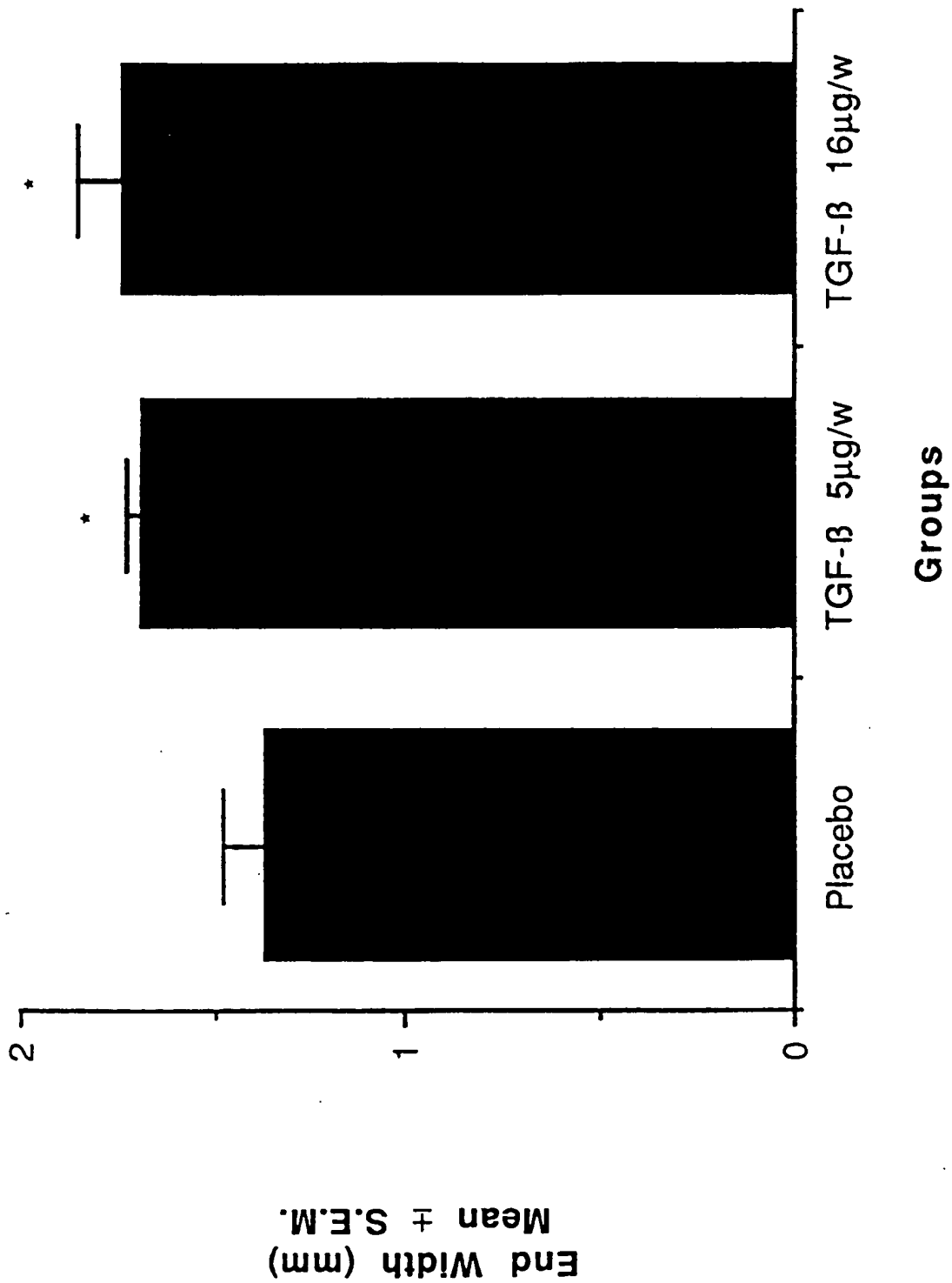

PERCENTAGE (%) OF WOUNDS WITH BONE FORMATION
80
60
40
20
0
42
70
DAYS

FIG. 1

FIG. 2


End Width (mm)
Mean ± S.E.M.
0
1
2
*
*
Placebo
TGF-β 5µg/w
TGF-β 16µg/w
Groups

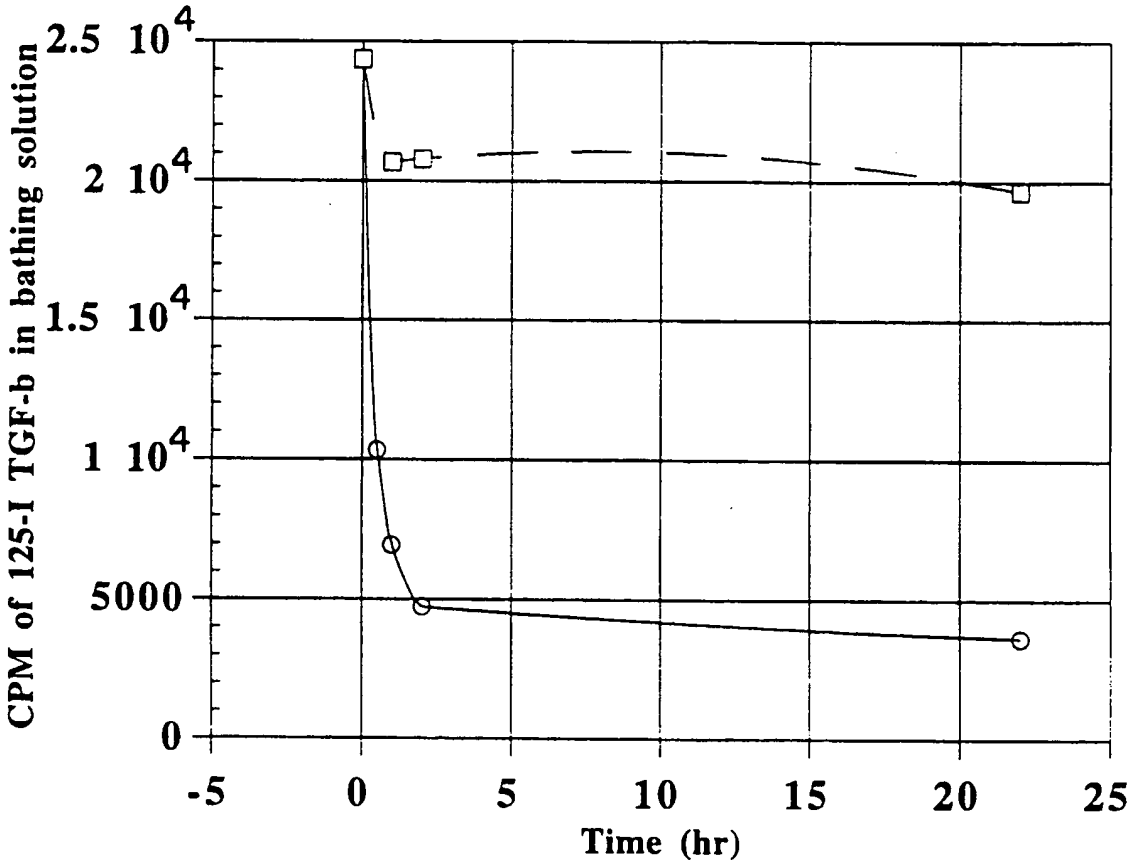

CPM of 125-I TGF-b in bathing solution
2.5 10^4
2 10^4
1.5 10^4
1 10^4
5000
0
-5
0
5
10
15
20
25
Time (hr)

FIG. 3

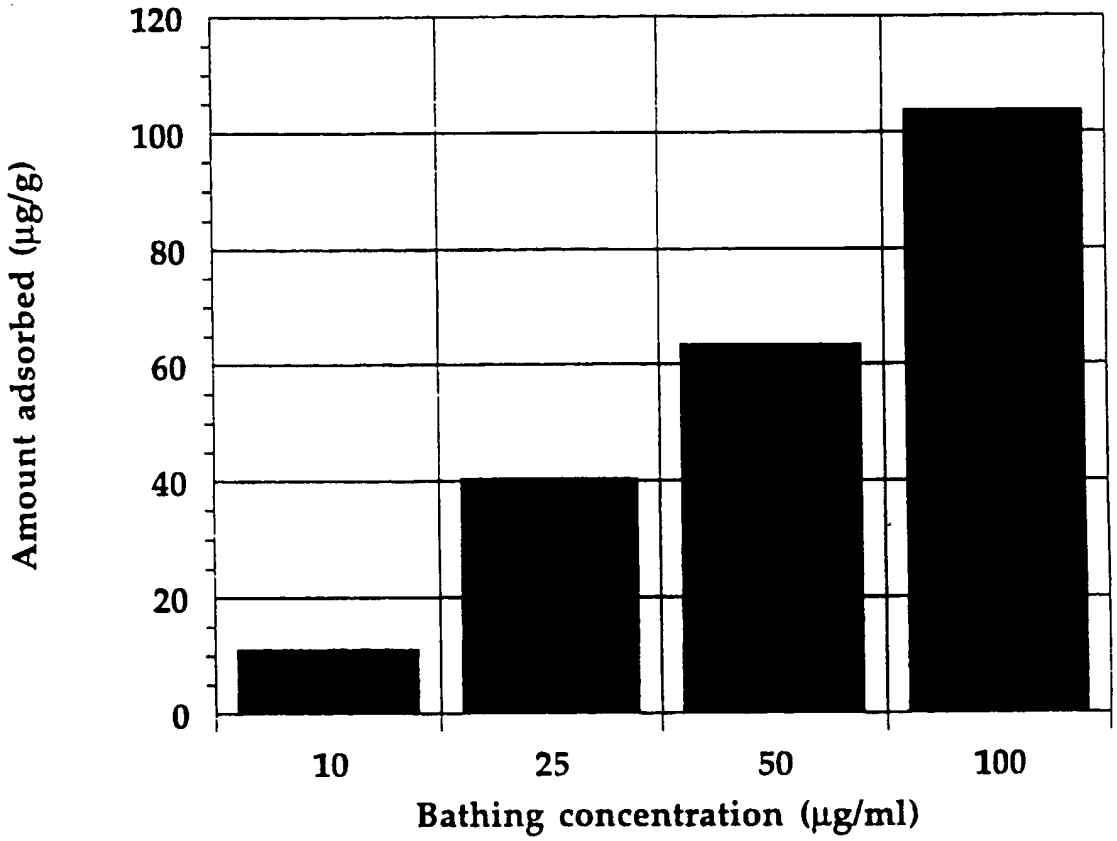

120
100
80
60
40
20
0
Amount adsorbed (μg/g)
10
25
50
100
Bathing concentration (μg/ml)

FIG. 4

FIG. 5

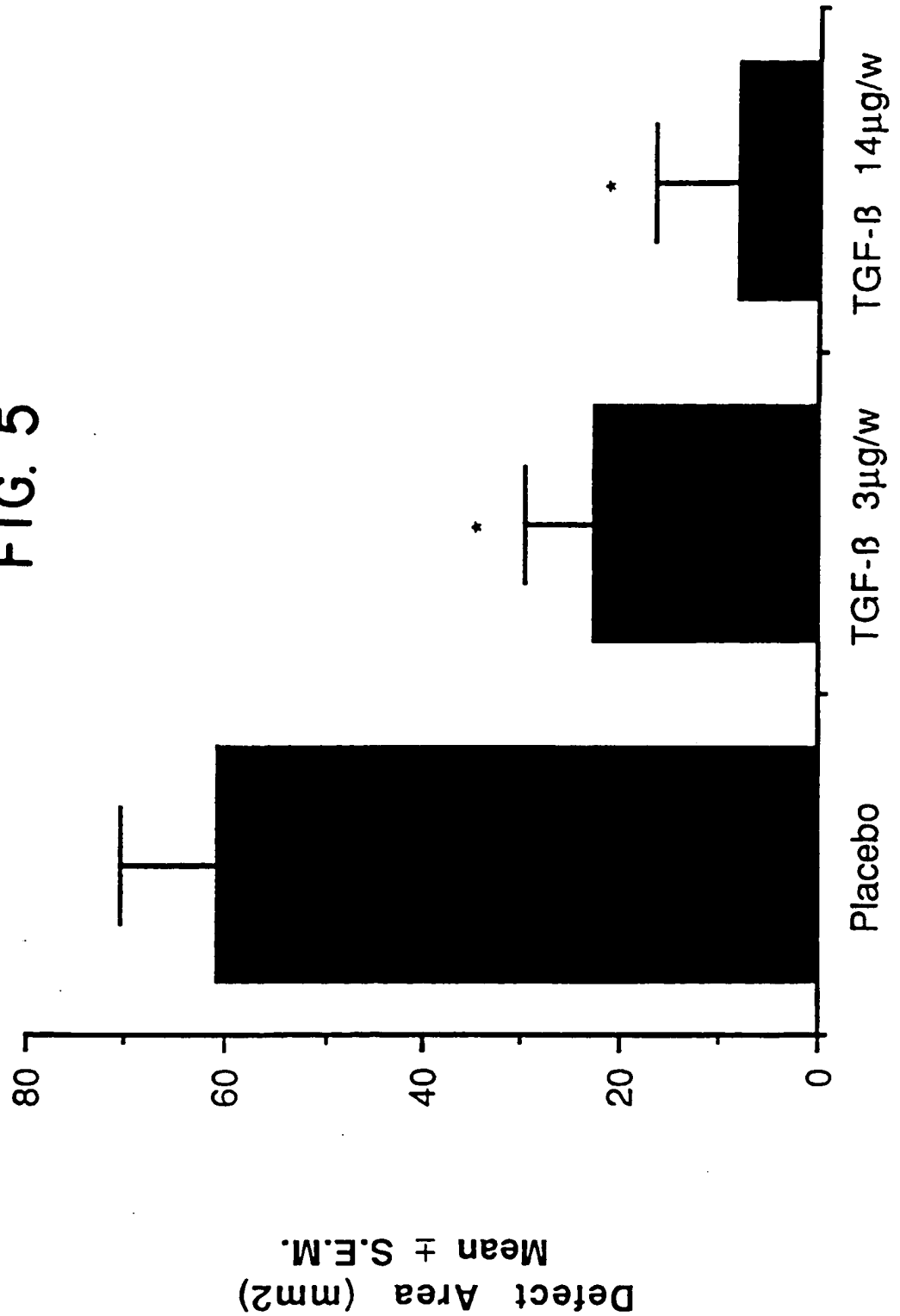

Defect Area (mm2)
Mean ± S.E.M.
80
60
40
20
0
*
*
Placebo
TGF-ß 3µg/w
TGF-ß 14µg/w
Groups

FIG. 6

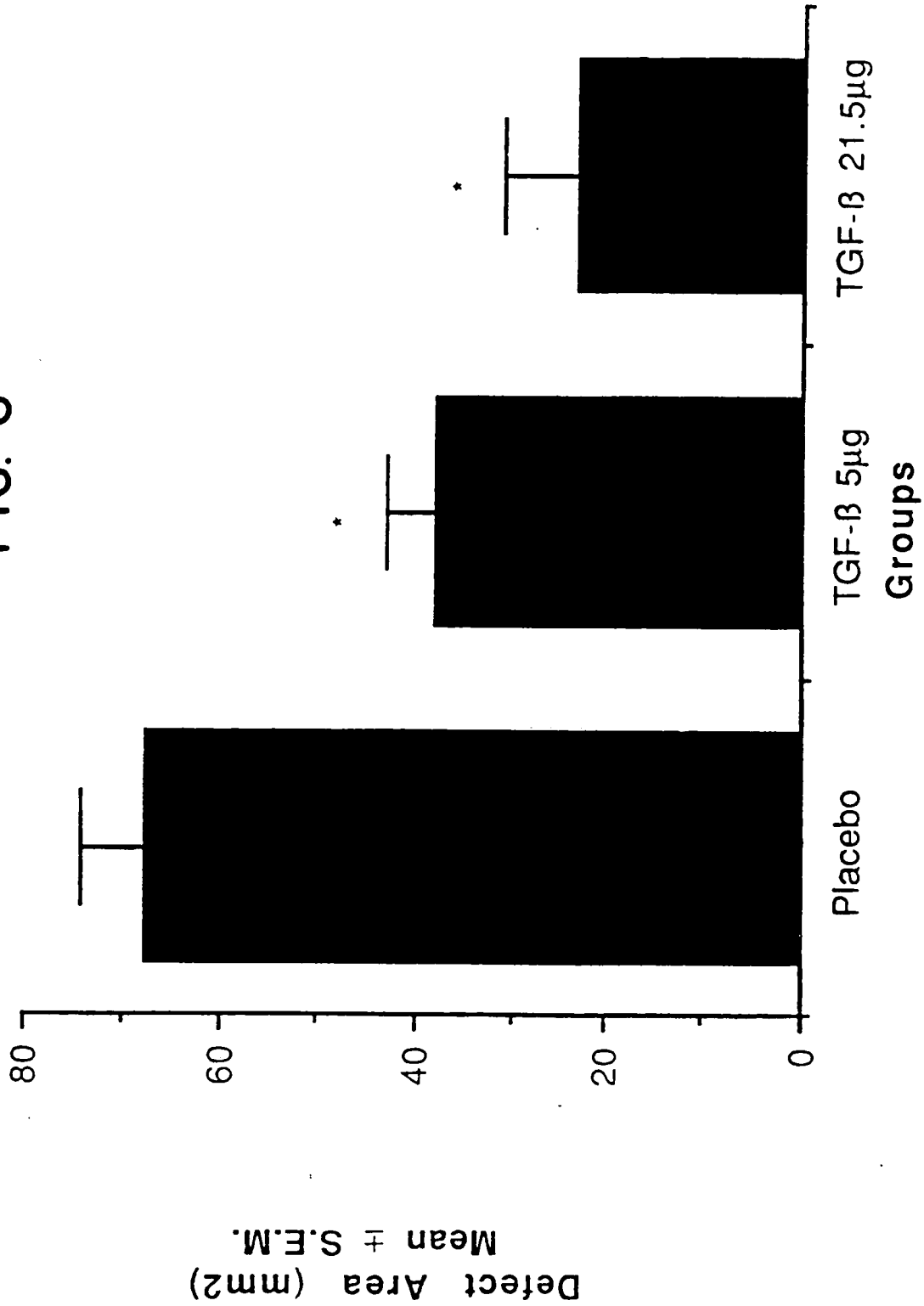

Defect Area (mm2)
Mean ± S.E.M.
0
20
40
60
80
Placebo
TGF-ß 5µg
TGF-ß 21.5µg
Groups
*
*

FIG. 7

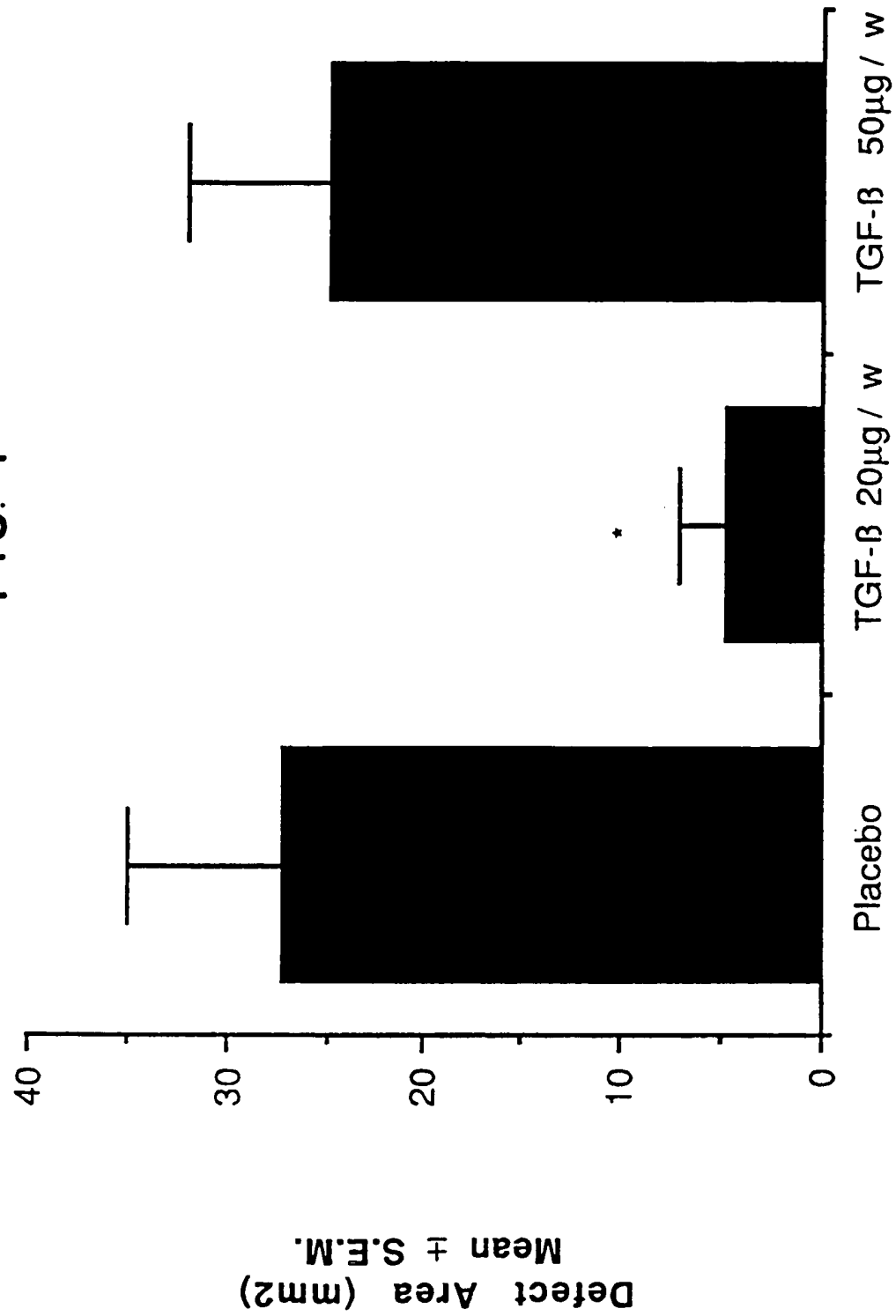

Defect Area (mm2)
Mean ± S.E.M.
40
30
20
10
0
*
Placebo
TGF-ß 20µg / w
TGF-ß 50µg / w
Groups

FIG. 8

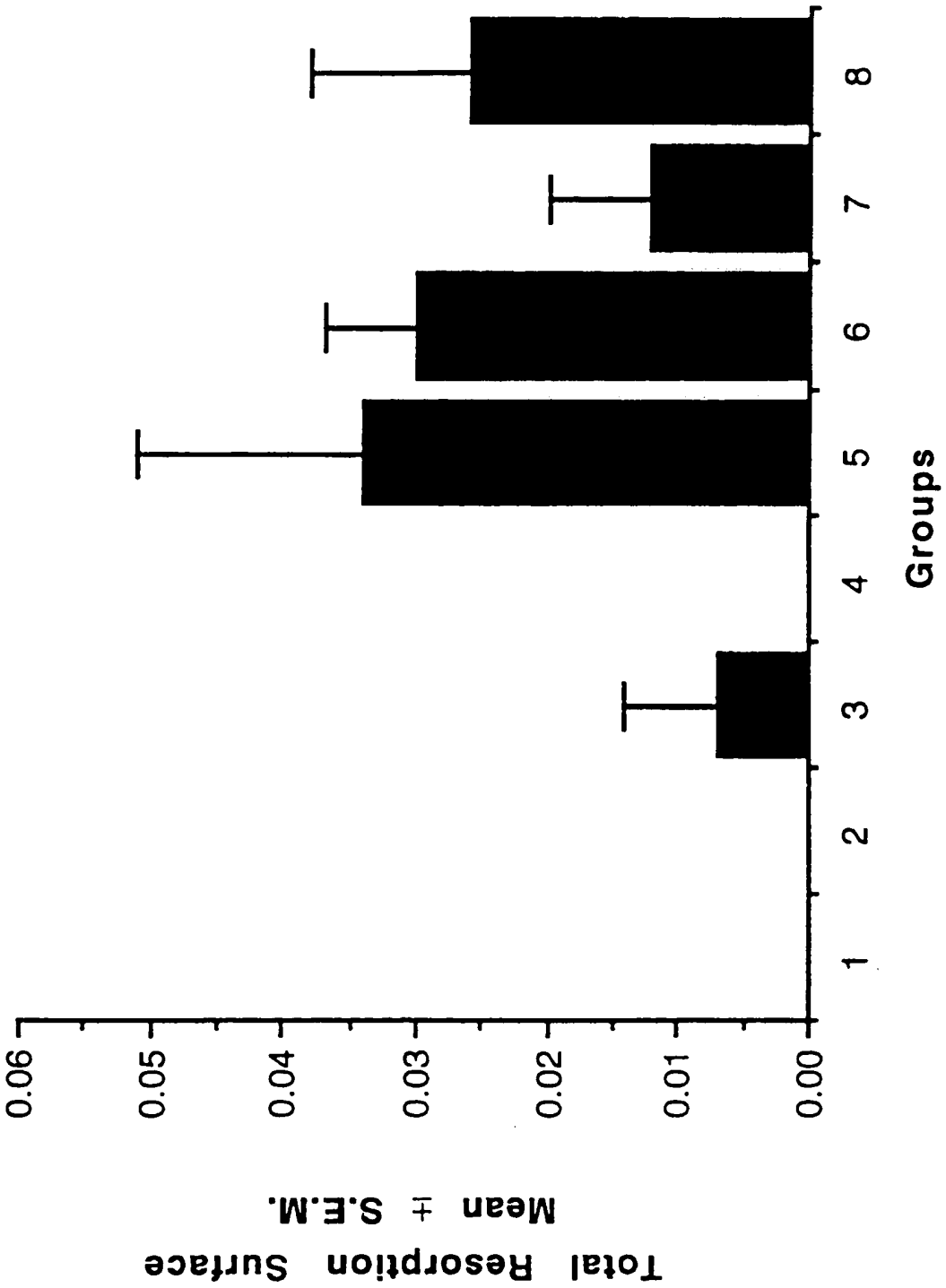

Total Resorption Surface
Mean ± S.E.M.
0.06
0.05
0.04
0.03
0.02
0.01
0.00
1
2
3
4
5
6
7
8
Groups

FIG. 9

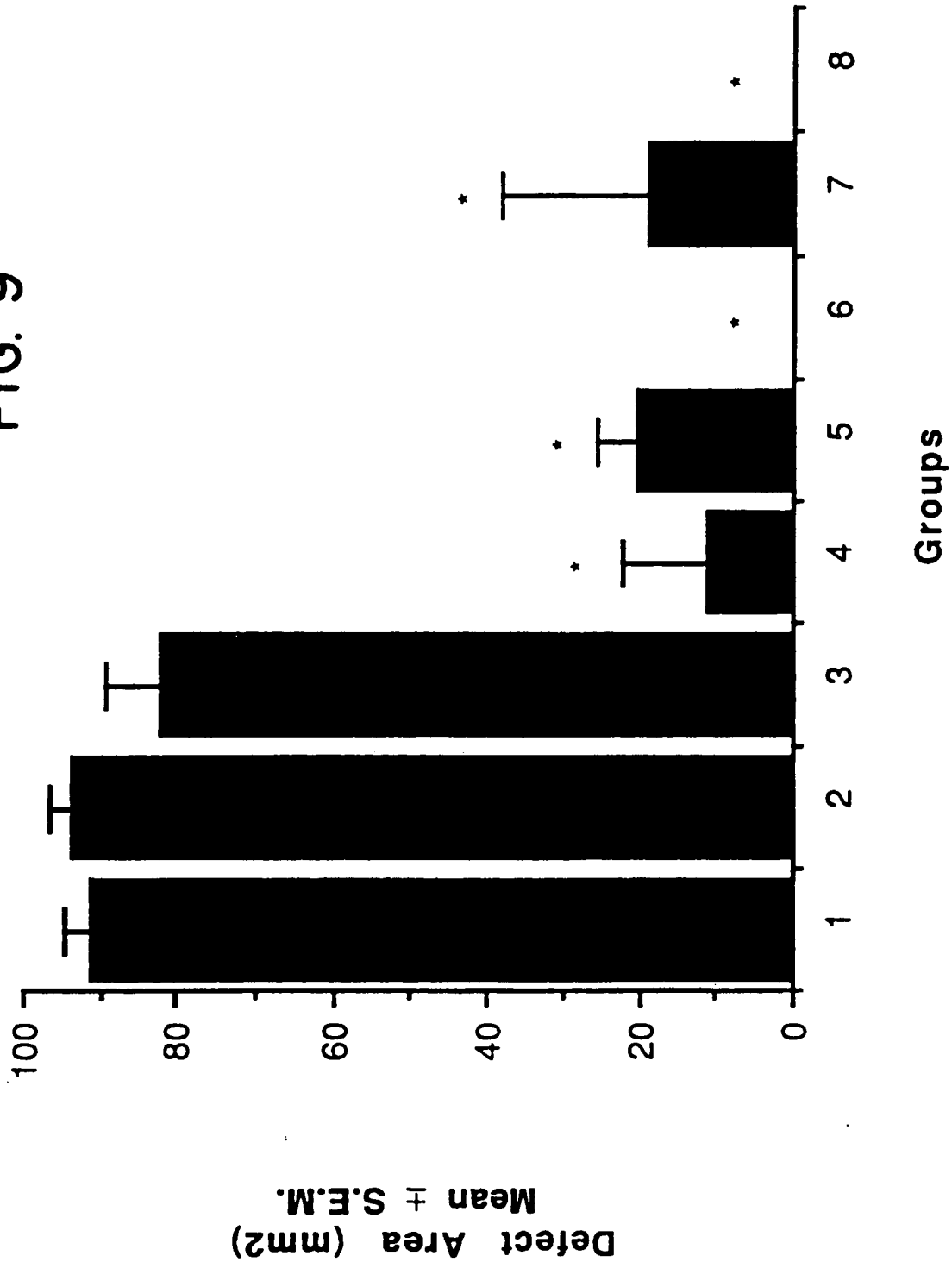
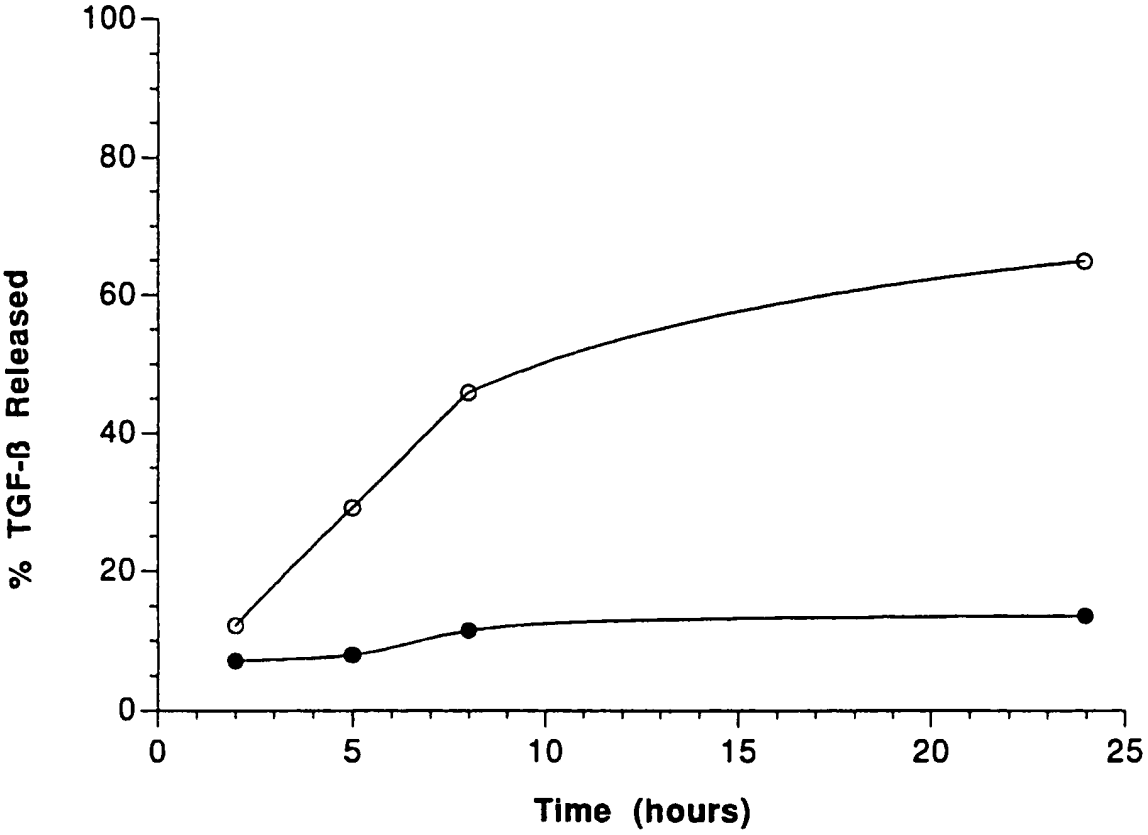


FIG. 10



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(54) Osteogenic devices

Osteogene Vorrichtungen

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The file contains technical information submitted after the application was filed and not included in this specification

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Description

[0001] This invention relates to osteogenic devices, to genes encoding proteins which can induce osteogenesis in mammals and methods for their production using recombinant DNA techniques, to a method of reproducibly purifying osteogenic protein from mammalian bone, and to bone and cartilage repair procedures using the osteogenic device.

[0002] Mammalian bone tissue is known to contain one or more proteinaceous materials, presumably active during growth and natural bone healing, which can induce a developmental cascade of cellular events resulting in endochondral bone formation. This active factor (or factors) has variously been referred to in the literature as bone morphogenetic or morphogenic protein, bone inductive protein, osteogenic protein, osteogenin, or osteoinductive protein.

[0003] The developmental cascade of bone differentiation consists of recruitment of mesenchymal cells, proliferation of progenitor cells, calcification of cartilage, vascular invasion, bone formation, remodeling, and finally marrow differentiation (Reddi (1981) Collagen Rel. Res. 1:209-226).

[0004] Though the precise mechanisms underlying these phenotypic transformations are unclear, it has been shown that the natural endochondral bone differentiation activity of bone matrix can be dissociatively extracted and reconstituted with inactive residual collagenous matrix to restore full bone induction activity (Sampath and Reddi, (1981) Proc. Natl. Acad. Sci. USA 78:7599-7603). This provides an experimental method for assaying protein extracts for their ability to induce endochondral bone in vivo.

[0005] This putative bone inductive protein has been shown to have a molecular mass of less than 50 kilodaltons (kD). Several species of mammals produce closely related protein as demonstrated by cross species implant experiments (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595).

[0006] The potential utility of these proteins has been widely recognized. It is contemplated that the availability of the protein would revolutionize orthopedic medicine, certain types of plastic surgery, and various periodontal and craniofacial reconstructive procedures.

[0007] The observed properties of these protein fractions have induced an intense research effort in various laboratories directed to isolating and identifying the pure factor or factors responsible for osteogenic activity. The current state of the art of purification of osteogenic protein from mammalian bone is disclosed by Sampath et al. (Proc. Natl. Acad. Sci. USA (1987) 80). Urist et al. (Proc. Soc. Exp. Biol. Med. (1984) 173:194-199) disclose a human osteogenic protein fraction which was extracted from demineralized cortical bone by means of a calcium chloride-urea inorganic-organic solvent mixture, and retrieved by differential precipitation in guanidine-hydrochloride and preparative gel electrophoresis. The authors report that the protein fraction has an amino acid composition of an acidic polypeptide and a molecular weight in a range of 17-18 kD.

[0008] Urist et al. (Proc. Natl. Acad. Sci. USA (1984) 81:371-375) disclose a bovine bone morphogenetic protein extract having the properties of an acidic polypeptide and a molecular weight of approximately 18 kD. The authors reported that the protein was present in a fraction separated by hydroxyapatite chromatography, and that it induced bone formation in mouse hindquarter muscle and bone regeneration in trephine defects in rat and dog skulls. Their method of obtaining the extract from bone results in ill-defined and impure preparations.

[0009] European Patent Application Serial No. 148,155, published October 7, 1985, purports to disclose osteogenic proteins derived from bovine, porcine, and human origin. One of the proteins, designated by the inventors as a P3 protein having a molecular weight of 22-24 kD, is said to have been purified to an essentially homogeneous state. This material is reported to induce bone formation when implanted into animals.

[0010] International Application No. PCT/087/01537, published January 14, 1988, discloses an impure fraction from bovine bone which has bone induction qualities. The named applicants also disclose putative bone inductive factors produced by recombinant DNA techniques. Four DNA sequences were retrieved from human or bovine genomic or cDNA libraries and apparently expressed in recombinant host cells. While the applicants stated that the expressed proteins may be bone morphogenic proteins, bone induction was not demonstrated. See also Urist et al., EP 0,212,474 entitled Bone Morphogenic Agents.

[0011] Wang et al. (Proc. Nat. Acad. Sci. USA (1988) 85: 9484-9488) discloses the purification of a bovine bone morphogenetic protein from guanidine extracts of demineralized bone having cartilage and bone formation activity as a basic protein corresponding to a molecular weight of 30 kD determined from gel elution. Purification of the protein yielded proteins of 30, 18 and 16 kD which, upon separation, were inactive. In view of this result, the authors acknowledged that the exact identity of the active material had not been determined.

[0012] Wozney et al. (Science (1988) 242: 1528-1534) discloses the isolation of full-length cDNA's encoding the human equivalents of three polypeptides originally purified from bovine bone. The authors report that each of the three recombinantly expressed human proteins are independently or in combination capable of inducing cartilage formation. No evidence of bone formation is reported.

[0013] It is an object of this invention to provide osteogenic devices comprising matrices containing dispersed osteogenic protein capable of bone induction in allogenic and xenogenic implants. Another object is to provide a reproducible method of isolating osteogenic protein from mammalian bone tissue. Another object is to characterize the protein

responsible for osteogenesis. Another object is to provide natural and recombinant osteogenic proteins capable of inducing endochondral bone formation in mammals, including humans. Yet another object is to provide genes encoding osteogenic proteins and methods for their production using recombinant DNA techniques. Another object is to provide methods for inducing cartilage formation.

[0014] These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

Summary of the Invention

[0015] This invention involves osteogenic devices which, when implanted in a mammalian body, can induce at the locus of the implant the full developmental cascade of endochondral bone formation and bone marrow differentiation. Suitably modified as disclosed herein, the devices also may be used to induce cartilage formation. The devices comprise a carrier material, referred to herein as a matrix, having the characteristics disclosed below, containing dispersed osteogenic protein either in its native form as purified from natural sources or produced using recombinant DNA techniques.

[0016] Key to these developments was the successful development of a protocol which results in retrieval of active, substantially pure osteogenic protein from mammalian bone, and subsequent elucidation of amino acid sequence and structure data of native osteogenic protein. The protein has a half-maximum bone forming activity of about 0.8 to 1.0 ng per mg of implant. The protein is believed to be a dimer. It appears not to be active when reduced. Various combinations of species of the proteins may exist as heterodimers or homodimers.

[0017] The invention provides native forms of osteogenic protein, extracted from bone or produced using recombinant DNA techniques. The substantially pure osteogenic protein may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology, and active truncated or mutated forms of native protein, no matter how derived. The naturally sourced osteogenic protein in its native form is glycosylated and has an apparent molecular weight of about 30 kD as determined by SDS-PAGE. When reduced, the 30 kD protein gives rise to two glycosylated polypeptide chains having apparent molecular weights of about 16 kD and 18 kD. In the reduced state, the 30 kD protein has no detectable osteogenic activity. The deglycosylated protein, which has osteogenic activity, has an apparent molecular weight of about 27 kD. When reduced, the 27 kD protein gives rise to the two deglycosylated polypeptides have molecular weights of about 14 kD to 16 kD.

[0018] Analysis of intact molecules and digestion fragments indicate that the native 30 kD osteogenic protein contains the following amino acid sequences (question marks indicate undetermined residues):

(1) S-F-D-A-Y-Y-C-S-G-A-C-Q-F-P-M-P-K;

(2) S-L-K-P-S-N-Y-A-T-I-Q-S-I-V;

(3) A-C-C-V-P-T-E-L-S-A-I-S-M-L-Y-L-D-E-N-E-K;

(4) M-S-S-L-S-I-L-F-F-D-E-N-K;

(5) S-Q-E-L-Y-V-D-F-Q-R;

(6) F-L-H-C-Q-F-S-E-R-N-S;

(7) T-V-G-Q-L-N-E-Q-S-S-E-P-N-I-Y;

(8) L-Y-D-P-M-V-V;

(9) V-G-V-V-P-G-I-P-E-P-C-C-V-P-E;

(10) V-D-F-A-D-I-G;

(11) V-P-K-P-C-C-A-P-T;

(12) I-N-I-A-N-Y-L;

(13) D-N-H-V-L-T-M-F-P-I-A-I-N;

(14) D-E-Q-T-L-K-K-A-R-R-K-Q-W-I-?-P;

(15) D-I-G-?-S-E-W-I-I-?-P;

(16) S-I-V-R-A-V-G-V-P-G-I-P-E-P-?-?-V;

(17) D-?-I-V-A-P-P-Q-Y-H-A-F-Y;

(18) D-E-N-K-N-V-V-L-K-V-Y-P-N-M-T-V-E;

(19) S-Q-T-L-Q-F-D-E-Q-T-L-K-?-A-R-?-K-Q;

(20) D-E-Q-T-L-K-K-A-R-R-K-Q-W-I-E-P-R-N-?-A-R-R-Y-L;

(21) A-R-R-K-Q-W-I-E-P-R-N-?-A-?-R-Y-?-?-V-D; and

(22) R-?-Q-W-I-E-P-?-N-?-A-?-?-Y-L-K-V-D-?-A-?-?-G.

[0019] The availability of the protein in substantially pure form, and knowledge of its amino acid sequence and other structural features, enable the identification, cloning, and expression of native genes which encode osteogenic proteins. When properly modified after translation, incorporated in a suitable matrix, and implanted as disclosed herein, these proteins are operative to induce formation of cartilage and endochondral bone.

[0020] Consensus DNA sequences designed as disclosed herein based on partial sequence data and observed homologies with regulatory proteins disclosed in the literature are useful as probes for extracting genes encoding osteogenic protein from genomic and cDNA libraries. One of the consensus sequences has been used to isolate a heretofore unidentified genomic DNA sequence, portions of which when ligated encode a protein having a region capable of inducing endochondral bone formation. This protein, designated OP1, has an active region having the sequence set forth below.

```
                1         10         20         30         40
OP1                  LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                           50         60         70
                YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
                    80         90        100
                ISVLYFDDSSNVILKKYRNMVVRACGCH
```

A longer active sequence is:

```
                                                  -5
                                                 HQRQA
          1         10        20        30        40
OP1       CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                         50        60        70
          YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
              80         90        100
          ISVLYFDDSSNVILKKYRNMVVRACGCH
```

Fig. 1A discloses the genomic DNA sequence of OP1.

[0021] The probes have also retrieved the DNA sequences identified in PCT/087/01537, referenced above, designated therein as BMPII(b) and BMPIII. The inventors herein have discovered that certain subparts of these genomic DNAs, and BMPIIa, from the same publication, when properly assembled, encode proteins (CBMPIIa, CBMPIIb, and CBMPIII) which have true osteogenic activity, i.e., induce the full cascade of events when properly implanted in a mammal leading to endochondral bone formation. These sequences are:

```
          1         10        20        30        40
CBMP-2a   CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
                         50        60        70
          HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
              80         90        100
          ISMLYLDENEKVVLKNYQDMVVEGCGCR

          1         10        20        30        40
CBMP-2b   CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
                         50        60        70
          HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
              80         90        100
          ISMLYLDEYDKVVLKNYQEMVVEGCGCR

          1         10        20        30        40
CBMP-3    CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPK
                         50        60        70
          SLKPSN--H-ATIQSIVRAVGVVPGIPEPCCVPEKMSS
              80         90        100
          LSILFFDENKNVVLKVYPNMTVESCACR
```

[0022] Thus, in view of this disclosure, skilled genetic engineers can isolate genes from cDNA or genomic libraries which encode appropriate amino acid sequences, and then can express them in various types of host cells, including both procaryotes and eucaryotes, to produce large quantities of active proteins capable of inducing bone formation in mammals including humans.

[0023] The substantially pure osteogenic proteins (i.e., naturally derived or recombinant proteins free of contaminating proteins having no osteoinductive activity) are useful in clinical applications in conjunction with a suitable delivery or support system (matrix). The matrix is made up of particles or porous materials. The pores must be of a dimension to permit progenitor cell migration and subsequent differentiation and proliferation. The particle size should be within the range of 70 - 850 µm, preferably 70 - 420 µm. It may be fabricated by close packing particulate material into a shape spanning the bone defect, or by otherwise structuring as desired a material that is biocompatible (non-inflammatory) and, biodegradable in vivo to serve as a "temporary scaffold" and substratum for recruitment of migratory progenitor cells, and as a base for their subsequent anchoring and proliferation. Currently preferred carriers include particulate, demineralized, guanidine extracted, species-specific (allogenic) bone, and particulate, deglycosylated (or HF treated), protein extracted, demineralized, xenogenic bone. Optionally, such xenogenic bone powder matrices also

may be treated with proteases such as trypsin. Other useful matrix materials comprise collagen, homopolymers and copolymers of glycolic acid and lactic acid, hydroxyapatite, tricalcium phosphate and other calcium phosphates.

[0024] The osteogenic proteins and implantable osteogenic devices enabled and disclosed herein will permit the physician to obtain optimal predictable bone formation to correct, for example, acquired and congenital craniofacial and other skeletal or dental anomalies (Glowacki et al. (1981) Lancet 1:959-963). The devices may be used to induce local endochondral bone formation in non-union fractures as demonstrated in animal tests, and in other clinical applications including periodontal applications where bone formation is required. Another potential clinical application is in cartilage repair, for example, in the treatment of osteoarthritis.

Brief Description of the Drawing

[0025] The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIGURE 1A represents the nucleotide sequence of the genomic copy of osteogenic protein "OP1" gene. The unknown region between 1880 and 1920 actually represents about 1000 nucleotides;

FIGURE 1B is a representation of the hybridization of the consensus gene/probe to the osteogenic protein "OP1" gene;

FIGURE 2 is a collection of plots of protein concentration (as indicated by optical absorption) vs elution volume illustrating the results of bovine osteogenic protein (BOP) fractionation during purification on heparin-Sepharose-I; HAP-Ultragel; sieving gel (Sephacryl 300); and heparin-Sepharose-II;

FIGURE 3 is a photographic reproduction of a Coomassie blue stained SDS polyacrylamide gel of the osteogenic protein under non-reducing (A) and reducing (B) conditions;

FIGURE 4 is a photographic reproduction of a Con A blot of an SDS polyacrylamide gel showing the carbohydrate component of oxidized (A) and reduced (B) 30 kD protein;

FIGURE 5 is a photographic reproduction of an autoradiogram of an SDS polyacrylamide gel of ^{125}I-labelled glycosylated (A) and deglycosylated (B) osteogenic protein under non-reducing (1) and reducing (2) conditions;

FIGURE 6 is a photographic reproduction of an autoradiogram of an SDS polyacrylamide gel of peptides produced upon the digestion of the 30 kD osteogenic protein with V-8 protease (B), Endo Lys C protease (C), pepsin (D), and trypsin (E). (A) is control;

FIGURE 7 is a collection of HPLC chromatograms of tryptic peptide digestions of 30 kD BOP (A), the 16 kD subunit (B), and the 18 kD subunit (C);

FIGURE 8 is an HPLC chromatogram of an elution profile on reverse phase C-18 HPLC of the samples recovered from the second heparin-Sepharose chromatography step (see FIGURE 2D). Superimposed is the percent bone formation in each fraction;

FIGURE 9 is a gel permeation chromatogram of an elution profile on TSK 3000/2000 gel of the C-18 purified osteogenic peak fraction. Superimposed is the percent bone formation in each fraction;

FIGURE 10 is a collection of graphs of protein concentration (as indicated by optical absorption) vs. elution volume illustrating the results of human protein fractionation on heparin-Sepharose I (A), HAP-Ultragel (B), TSK 3000/2000 (C), and heparin-Sepharose II (D). Arrows indicate buffer changes;

FIGURE 11 is a graph showing representative dose response curves for bone-inducing activity in samples from various purification steps including reverse phase HPLC on C-18 (A), Heparin-Sepharose II (B), TSK 3000 (C), HAP-ultragel (D), and Heparin-Sepharose I (E);

FIGURE 12 is a bar graph of radiomorphometric analyses of feline bone defect repair after treatment with an osteogenic device (A), carrier control (B), and demineralized bone (C);

FIGURE 13 is a schematic representation of the DNA sequence and corresponding amino acid sequence of a consensus gene/probe for osteogenic protein (COPO);

FIGURE 14 is a graph of osteogenic activity vs. increasing molecular weight showing peak bone forming activity in the 30 kD region of an SDS polyacrylamide gel;

FIGURE 15 is a photographic representation of a Coomassie blue stained SDS gel showing gel purified subunits of the 30 kD protein;

FIGURE 16 is a pair of HPLC chromatograms of Endo Asp N proteinase digests of the 18 kD subunit (A) and the 16 kD subunit (B);

FIGURE 17 is a photographic representation of the histological examination of bone implants in the rat model: carrier alone (A); carrier and glycosylated osteogenic protein (B); and carrier and deglycosylated osteogenic protein (C). Arrows indicate osteoblasts;

FIGURE 18 is a graph illustrating the activity of xenogenic matrix (deglycolylated bovine matrix); and

FIGURES.19A and 19B are bar graphs showing the specific activity of naturally sourced OP before and after gel elution as measured by calcium content vs. increasing concentrations of proteins (dose curve, in ng).

Description

[0026] Purification protocols have been developed which enable isolation of the osteogenic protein present in crude protein extracts from mammalian bone. While each of the separation steps constitute a known separation technique, it has been discovered that the combination of a sequence of separations exploiting the protein's affinity for heparin and for hydroxyapatite (HAP) in the presence of a denaturant such as urea is key to isolating the pure protein from the crude extract. These critical separation steps are combined with separations on hydrophobic media, gel exclusion chromatography, and elution form SDS PAGE.

[0027] The isolation procedure enables the production of significant quantities of substantially pure osteogenic protein from any mammalian species, provided sufficient amounts of fresh bone from the species is available. The empirical development of the procedure, coupled with the availability of fresh calf bone, has enabled isolation of substantially pure bovine osteogenic protein (BOP). BOP has been characterized significantly as set forth below; its ability to induce cartilage and ultimately endochondral bone growth in cat, rabbit, and rat have been studied; it has been shown to be able to induce the full developmental cascade of bone formation previously ascribed to unknown protein or proteins in heterogeneous bone extracts; and it may be used to induce formation of endochondral bone in orthopedic defects including non-union fractures. In its native form it is a glycosylated, dimeric protein. However, it is active in deglycosylated form. It has been partially sequenced. Its primary structure includes the amino acid sequences set forth herein.

[0028] Elucidation of the amino acid sequence of BOP enables the construction of pools of nucleic acid probes encoding peptide fragments. Also, a consensus nucleic acid sequence designed as disclosed herein based on the amino acid sequence data, inferred codons for the sequences, and observation of partial homology with known genes, also has been used as a probe. The probes may be used to isolate naturally occuring cDNAs which encode active mammalian osteogenic proteins (OP) as described below using standard hybridization methodology. The mRNAs are present in the cytoplasm of cells of various species which are known to synthesize osteogenic proteins. Useful cells harboring the mRNAs include, for example, osteoblasts from bone or osteosarcoma, hypertrophic chondrocytes, and stem cells. The mRNAs can be used to produce cDNA libraries. Alternatively, relevant DNAs encoding osteogenic protein may be retrieved from cloned genomic DNA libraries from various mammalian species.

[0029] These discoveries enable the construction of DNAs encoding totally novel, non-native protein constructs which individually, and combined are capable of producing true endochondral bone. They also permit expression of the natural material, truncated forms, muteins, analogs, fusion proteins, and various other variants and constructs, from cDNAs retrieved from natural sources or synthesized using the techniques disclosed herein using automated, commercially available equipment. The DNAs may be expressed using well established recombinant DNA technologies in procaryotic or eucaryotic host cells, and may be oxidized and refolded in vitro if necessary for biological activity.

[0030] The isolation procedure for obtaining the protein from bone, the retrieval of an osteogenic protein gene, the design and production of recombinant protein, the nature of the matrix, and other material aspects concerning the nature, utility, how to make, and how to use the subject matter claimed herein will be further understood from the following, which constitutes the best method currently known for practicing the various aspects of the invention.

A - PURIFICATION OF BOP

A1. Preparation of Demineralized Bone

[0031] Demineralized bovine bone matrix is prepared by previously published procedures (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595). Bovine diaphyseal bones (age 1-10 days) are obtained from a local slaughterhouse and used fresh. The bones are stripped of muscle and fat, cleaned of periosteum, demarrowed by pressure with cold water, dipped in cold absolute ethanol, and stored at -20°C. They are then dried and fragmented by crushing and pulverized in a large mill. Care is taken to prevent heating by using liquid nitrogen. The pulverized bone is milled to a particle size between 70-420 μm and is defatted by two washes of approximately two hours duration with three volumes of chloroform and methanol (3:1). The particulate bone is then washed with one volume of absolute ethanol and dried over one volume of anhydrous ether. The defatted bone powder (the alternative method is to obtain Bovine Cortical Bone Powder (75-425 μm) from American Biomaterials) is then demineralized with 10 volumes of 0.5 N HC1 at 4°C for 40 min., four times. Finally, neutralizing washes are done on the demineralized bone powder with a large volume of water.

A2. Dissociative Extraction and Ethanol Precipitation

[0032] Demineralized bone matrix thus prepared is dissociatively extracted with 5 volumes of 4 M guanidine-HCl (Gu-HCl), 50mM Tris-HCl, pH 7.0, containing protease inhibitors (5 mM benzamidine, 44 mM 6-aminohexanoic acid, 4.3 mM N-ethylmaleimide, 0.44 mM phenylmethylsulfonyfluoride) for 16 hr. at 4°C. The suspension is filtered. The supernatant is collected and concentrated to one volume using an ultrafiltration hollow fiber membrane (Amicon, YM-10). The concentrate is centrifuged (8,000 x g for 10 min. at 4°C), and the supernatant is then subjected to ethanol precipitation. To one volume of concentrate is added five volumes of cold (-70°C) absolute ethanol (100%), which is then kept at -70°C for 16 hrs. The precipitate is obtained upon centrifugation at 10,000 x g for 10 min. at 4°C. The resulting pellet is resuspended in 4 l of 85% cold ethanol incubated for 60 min. at -70°C and recentrifuged. The precipitate is again resuspended in 85% cold ethanol (2 l), incubated at -70°C for 60 min. and centrifuged. The precipitate is then lyophilized.

A3. Heparin-Sepharose Chromatography I

[0033] The ethanol precipitated, lyophilized, extracted crude protein is dissolved in 25 volumes of 6 M urea, 50 mM Tris-HCl, pH 7.0 (Buffer A) containing 0.15 M NaCl, and clarified by centrifugation at 8,000 x g for 10 min. The heparin-Sepharose is column-equilibrated with Buffer A. The protein is loaded onto the column and after washing with three column volume of initial buffer (Buffer A containing 0.15 M NaCl), protein is eluted with Buffer A containing 0.5 M NaCl. The absorption of the eluate is monitored continuously at 280 nm. The pool of protein eluted by 0.5 M NaCl (approximately 1 column volumes) is collected and stored at 4°C.

[0034] As shown in FIGURE 2A, most of the protein (about 95%) remains unbound. Approximately 5% of the protein is bound to the column. The unbound fraction has no bone inductive activity when bioassayed as a whole or after a partial purification through Sepharose CL-6B.

A4. Hydroxyapaptite-Ultrogel Chromatography

[0035] The volume of protein eluted by Buffer A containing 0.5 M NaCl from the heparin-Sepharose is applied directly to a column of hydroxyapaptite-ultrogel (HAP-ultrogel) (LKB Instruments), equilibrated with Buffer A containing 0.5 M NaCl. The HAP-ultrogel is treated with Buffer A containing 500 mM Na phosphate prior to equilibration. The unadsorbed protein is collected as an unbound fraction, and the column is washed with three column volumes of Buffer A containing 0.5 M NaCl. The column is subsequently eluted with Buffer A containing 100 mM Na Phosphate (FIGURE 2B).

[0036] The eluted component can induce endochondral bone as measured by alkaline phosphatase activity and histology. As the biologically active protein is bound to HAP in the presence of 6 M urea and 0.5 M NaCl, it is likely that the protein has an affinity for bone mineral and may be displaced only by phosphate ions.

A5. Sephacryl S-300 Gel Exclusion Chromatography

[0037] Sephacryl S-300 HR (High Resolution, 5 cm x 100 cm column) is obtained from Pharmacia and equilibrated with 4 M guanidine-HCl, 50 mM Tris-HCl, pH 7.0. The bound protein fraction from HA-ultrogel is concentrated and exhanged from urea to 4 M guanidine-HCl, 50 mM Tris-HCl, pH 7.0 via an Amicon ultrafiltration YM-10 membrane. The solution is then filtered with Schleicher and Schuell CENTREX disposable microfilters. A sample aliquot of ap-

proximately 15 ml containing approximately 400 mg of protein is loaded onto the column and then eluted with 4 M guanidine-HCl, 50 mM Tris-HCl, pH 7.0, with a flow rate of 3 ml/min; 12 ml fractions are collected over 8 hours and the concentration of protein is measured at A_{280}nm (FIGURE 2C). An aliquot of the individual fractions is bioassayed for bone formation. Those fractions which have shown bone formation and migrate with an apparent molecular weight of less than 35 kD are pooled and concentrated via an Amicon ultrafiltration system with YM-10 membrane.

A6. Heparin-Sepharose Chromatography-II

[0038] The pooled osteo-inductive fractions obtained from gel exclusion chromatography are dialysed extensively against distilled water (dH_2O and then against 6 M urea, 50 mM Tris-HCl, pH 7.0 (Buffer A) containing 0.1 M NaCl. The dialysate is then cleared through centrifugation. The sample is applied to the heparin-sepharose column (equilibrated with the same buffer). After washing with three column volumes of initial buffer, the column is developed sequentially with Buffer B containing 0.15 M NaCl, and 0.5 M NaCl (FIGURE 2D). The protein eluted by 0.5 M NaCl is collected and dialyzed extensively against distilled water. It is then dialyzed against 30% acetonitrile, 0.1% TFA at 4°C.

A7. Reverse Phase HPLC

[0039] The protein is further purified by C-18 Vydac silica-based HPLC column chromatography (particle size 5 µm; pore size 300 A). The osteoinductive fraction obtained from heparin-sepharose-II chromatograph is loaded onto the column, and washed in 0.1% TFA, 10% acetonitrile for five min. As shown in FIGURE 8, the bound proteins are eluted with a linear gradient of 10-30% acetonitrile over 15 min., 30-50% acetonitrile over 60 min, and 50-70% acetonitrile over 10 min at 22°C with a flow rate of 1.5 ml/min and 1.4 ml samples are collected in polycarbonate tubes. Protein is monitored by absorbance at A_{214} nm. Column fractions are tested for the presence of osteoinductive activity, and concanavalin A-blottable proteins. These fractions are then pooled, and characterized biochemically for the presence of 30 kD protein by autoradiography, concanavalin A blotting, and Coomassie blue dye staining. They are then assayed for in vivo osteogenic activity. Biological activity is not found in the absence of 30 kD protein.

A8. Gel Elution

[0040] The glycosylated or deglycosylated protein is eluted from SDS gels (0.5 mm thickness) for further characterization. ^{125}I-labelled 30 kD protein is routinely added to each preparation to monitor yields. TABLE 1 shows the various elution buffers that have been tested and the yields of ^{125}I-labelled protein.

TABLE 1

Elution of 30 kD Protein from SDS Gel	
Buffer	% Eluted
(1) dH_2O	22
(2) 4 M Guanidine-HCl, Tris-HCl, pH 7.0	2
(3) 4 M Guanidine-HCl, Tris-HCl, pH 7.0, 0.5% Triton x 100	93
(4) 0.1% SDS, Tris-HCl, pH 7.0	98

[0041] TABLE 2 lists the steps used to isolate the 30 kD or deglycosylated 27 kD gel-bound protein. The standard protocol uses diffusion elution using 4M guanidine-HCl containing 0.5% Triton x 100 in Tris-HCl buffer or in Tris-HCl buffer containing 0.1% SDS to achieve greater than 95% elution of the protein from the 27 or 30 kD region of the gel for demonstration of osteogenic activity in vivo as described in later section.

TABLE 2

Preparation of Gel Eluted Protein	
(C-18 Pool or deglycoslated protein plus ^{125}I-labelled 30 kD protein)	
1.	Dry using vacuum centrifugation;
2.	Wash pellet with H_2O;
3.	Dissolve pellet in gel sample buffer (no reducing agent);
4.	Electrophorese on pre-electrophoresed 0.5 mm mini gel;
5.	Cut out 27 or 30 kD protein;

TABLE 2 (continued)

Preparation of Gel Eluted Protein	
(C-18 Pool or deglycoslated protein plus ^{125}I-labelled 30 kD protein)	
6.	Elute from gel with 0.1% SDS, 50mM Tris-HCl, pH 7.0;
7.	Filter through Centrex membrane;
8.	Concentrate and wash with water in Centricon tube (10 kD membrane).

[0042] The overall yield of labelled 30 kD protein from the gel elution protocol is 50 - 60% of the loaded sample. Most of the loss occurs in the electrophoresis step, due to protein aggregation and/or smearing.

[0043] The yield is 0.5 to 1.0 µg substantially pure osteogenic protein per kg of bone.

A9. Isolation of the 16 kD and 18 kD Species

[0044] TABLE 3 summarizes the procedures involved in the preparation of the subunits. Approximately 10 µg of gel eluted 30 kD protein (FIGURE 3) is carboxymethylated and electrophoresed on an SDS-gel. The sample contains ^{125}I-label to trace yields and to use as an indicator for slicing the 16 kD and 18 kD regions from the gel. FIGURE 15 shows a Coomassie blue stained gel of gel-purified 16 kD and 18 kD proteins.

TABLE 3

Isolation of the Subunits of the 30 kD protein	
(C-18 pool plus ^{125}I-labeled 30 kD protein)	
1.	Electrophorese on SDS gel.
2.	Cut out 30 kD protein.
3.	Elute with 0.1% SDS, 50 mM Tris-HCl, pH 7.0.
4.	Concentrate and wash with H_2O in Centricon tube (10 kD membranes).
5.	Electrophorese reduced sample on SDS gel.
6.	Cut out the 16 kD and 18 kD subunits.
7.	Elute with 0.1% SDS, 50 mM Tris-HCl, pH 7.0.
8.	Concentrate and wash with H_2O in Centricon tubes.
9.	Reduce and carboxymethylate in 1% SDS, 0.4 M Tris-HCl, pH 8.5.
10.	Concentrate and wash with H_2O in Centricon tube.

B. Biological Characterization of BOP

B1. Gel Slicing:

[0045] Gel slicing experiments confirm that the isolated 30 kD protein is the protein responsible for osteogenic activity.

[0046] Gels from the last step of the purification are sliced. Protein in each fraction is extracted in 15 mM Tris-HCl, pH 7.0 containing 0.1% SDS or in buffer containing 4 M guanidine-HCl, 0.5% non-ionic detergent (Triton x 100), 50 mM Tris-HCl. The extracted proteins are desalted, concentrated, and assayed for endochondral bone formation activity. The results are set forth in FIGURE 14. From this figure it is clear that the majority of osteogenic activity is due to protein at 30 kD region of the gel. Activity in higher molecular weight regions is apparently due to protein aggregation. These protein aggregates, when reduced, yields the 16 kD and 18 kD species discussed above.

B2. Con A-Sepharose Chromatography;

[0047] A sample containing the 30 kD protein is solubilized using 0.1% SDS, 50 mM Tris-HCl, and is applied to a column of concanavalin A (Con A)-Sepharose equilibrated with the same buffer. The bound material is eluted in SDS Tris-HCl buffer containing 0.5 M alpha-methyl mannoside. After reverse phase chromatography of both the bound and unbound fractions, Con A-bound materials, when implanted, result in extensive bone formation. Further characterization of the bound materials show a Con A-blottable 30 kD protein. Accordingly, the 30 kD glycosylated protein is responsible for the bone forming activity.

B3. Gel Permeation Chromatography:

[0048] TSK-3000/2000 gel permeation chromatography in guanidine-HCl alternately is used to achieve separation of the high specific activity fraction obtained from C-18 chromatography (FIGURE 9). The results demonstrate that the peak of bone inducing activity elutes in fractions containing substantially pure 30 kD protein by Coomassie blue staining. When this fraction is iodinated and subjected to autoradiography, a strong band at 30 kD accounts for 90% of the iodinated proteins. The fraction induces bone formation in vivo at a dose of 50 to 100 ng per implant.

B4. Structural Requirements for Biological Activity

B4-1 Activity after Digestion

[0049] Although the role of 30 kD osteogenic protein is clearly established for bone induction, through analysis of proteolytic cleavage products we have begun to search for a minimum structure that is necessary for activity in vivo. The results of cleavage experiments demonstrate that pepsin treatment fails to destroy bone inducing capacity, whereas trypsin or CNBr completely abolishes the activity.

[0050] An experiment is performed to isolate and identify pepsin digested product responsible for biological activity. The sample used for pepsin digestion was 20% - 30% pure. The buffer used is 0.1% TFA in water. The enzyme to substrate ratio is 1:10. A control sample is made without enzyme. The digestion mixture is incubated at room temperature for 16 hr. The digested product is then separated in 4 M guanidine-HCl using gel permeation chromatography, and the fractions are prepared for in vivo assay. The results demonstrate that active fractions from gel permeation chromotography of the pepsin digest correspond to peptides having an apparent molecular weight range of 8 kD - 10 kD.

B4-2 Unglycosylated Protein is Active

[0051] In order to understand the importance of the carbohydrates moiety with respect to osteogenic activity, the 30 kD protein has been chemically deglycosylated using HF (see below). After analyzing an aliquot of the reaction product by Con A blot to confirm the absence of carbohydrate, the material is assayed for its activity in vivo. The bioassay is positive (i.e., the deglycosylated protein produces a bone formation response as determined by histological examination shown in FIGURE 17C), demonstrating that exposure to HF did not destroy the biological function of the protein, and thus that the OP does not require carboyhdrate for biological activity. In addition, the specific activity of the deglycosylated protein is approximately the same as that of the native glycosylated protein.

B5. Specific Activity of BOP

[0052] Experiments were performed 1) to determine the half maximal bone-inducing activity based on calcium content of the implant; 2) to estimate proteins at nanogram levels using a gel scanning method; and 3) to establish dose for half maximal bone inducing activity for gel eluted 30 kD BOP. The results demonstrate that gel eluted substantially pure 30 kD osteogenic protein induces bone at less than 5 ng per implant and exhibits half maximal bone differentiation activity at 20 ng per implant (approx. 25 mg). The purification data suggest that osteogenic protein has been purified from bovine bone to 367,307 fold after the final gel elution step with a specific activity of 47,750 bone forming units per mg of protein.

B5(a)Half Maximal Bone Differentiation Activity

[0053] The bone inducing activity is determined biochemically by the specific activity of alkaline phosphatase and calcium content of the day 12 implant. An increase in the specific activity of alkaline phosphatase indicates the onset of bone formation. Calcium content, on the other hand, is proportional to the amount of bone formed in the implant. The bone formation is therefore calculated by determining calcium content of the implant on day 12 in rats and expressed as bone forming units, which represent the amount that exhibits half maximal bone inducing activity compared to rat demineralized bone matrix. Bone induction exhibited by intact demineralized rat bone matrix is considered to be the maximal bone-differentiation activity for comparison.

B5(b) Protein Estimation Using Gel Scanning Techniques

[0054] A standard curve is developed employing known amounts of a standard protein, bovine serum albumin. The protein at varying concentration (50-300 ng) is loaded on a 15% SDS gel, electrophoresed, stained in comassie and destained. The gel is scanned at predetermined settings using a gel scanner at 580 nm. The area covered by the

protein band is calculated and a standard curve against concentrations of protein is constructed. A sample with an unknown protein concentration is electrophoresed with BSA as a standard. The lane containing the unknown sample is scanned, and the concentration of protein is determined from the area under the curve.

B5(c)Gel Elution and Specific Activity

[0055] An aliquot of C-18 highly purified active fraction is subjected to SDS gel and sliced according to molecular weights described in FIGURE 14. Proteins are eluted from the slices in 4 M guanidine-HCl containing 0.5% Triton X-100, desalted, concentrated and assayed for endochondral bone forming activity as determined by calcium content. The C-18 highly active fractions and gel eluted substantially pure 30 kD osteogenic protein are implanted in varying concentrations in order to determine the half maximal bone inducing activity.

[0056] FIGURE 14 shows that the bone inducing activity is due to proteins eluted in the 28-34 kD region. The recovery of activity after the gel elution step is determined by calcium content. FIGURES 19A and 19B represent the bone inducing activity for the various concentrations of 30 kD protein before and after gel elution as estimated by calcium content. The data suggest that the half maximal activity for 30 kD protein before gel elution is 69 ng per 25 mg implant and is 21 ng per 25 mg implant after elution. TABLE 4 describes the yield, total specific activity, and fold purification of osteogenic protein at each step during purification. Approximately 500 ug of heparin sepharose I fraction, 130-150 ug of the HA ultrogel fraction, 10-12 ug of the gel filtration fraction, 4-5 ug of the heparin sepharose II fraction, 0.4-0.5 ug of the C-18 highly purified fraction, and 20-25 ng of the gel eluted, substantially purified fraction is needed per 25 mg of implant for unequivocal bone formation for half maximal activity. Thus, 0.8-1.0 ng purified osteogenic protein per mg. of implant is required to exhibit half maximal bone differentiation activity in vivo.

TABLE 4

PURIFICATION OF BOP				
Purification Steps	Protein (mg.)	Biological Activity Units*	Specific Activity Units/mg.	Purification Fold
Ethanol Precipitate**	30,000#	4,000	0.13	1
Heparin Sepharose I	1,200#	2,400	2.00	15
HA-Ultrogel	300#	2,307	7.69	59
Gel filtration	20#	1,600	80.00	615
Heparin Sepharose II	5#	1,000	200.00	1,538.
C-18 HPLC	0.070@	150	2,043.00	15,715
Gel elution	0.004@	191	47,750.00	367,307

Values are calculated from 4 kg of bovine bone matrix (800 g of demineralized matrix).

* One unit of bone forming activity is defined as the amount that exhibits half maximal bone differentiation activity compared to rat demineralized bone matrix, as determined by calcium content of the implant on day 12 in rats.

Proteins were measured by absorbance at 280 nm.

@ Proteins were measured by gel scanning method compared to known standard protein, bovine serum albumin.

** Ethanol-precipitated guanidine extract of bovine bone is a weak inducer of bone in rats, possibly due to endogenous inhibitors. This precipitate is subjected to gel filtration and proteins less than 50 kD were separated and used for bioassay.

C. CHEMICAL CHARACTERIZATION OF BOP

C1. Molecular Weight and Structure

[0057] Electrophoresis of the proteins after the final purification step on non-reducing SDS polyacrylamide gels reveals a diffuse band at about 30 kD as detected by both Coomassie blue staining (FIGURE 3A) and autoradiography.

[0058] In order to extend the analysis of BOP, the protein was examined under reducing conditions. FIGURE 3B shows an SDS gel of BOP in the presence of dithiothreitol. Upon reduction, 30 kD BOP yields two species which are stained with Coomassic blue dye: a 16 kD species and an 18 kD species. Reduction causes loss of biological activity. The two reduced BOP species have been analyzed to determine if they are structurally related. Comparison of the amino acid composition and peptide mapping of the two species (as disclosed below) shows little differences, indicating that the native protein may comprise two chains having significant homology.

C2. Presence of Carbohydrate

[0059] The 30 kD protein has been tested for the presence of carbohydrate by Con A blotting after SDS-PAGE and transfer to nitrocellulose paper. The results demonstrate that the 30 kD protein has a high affinity for Con A, indicating that the protein is glycosylated (FIGURE 4A). In addition, the Con A blots provide evidence for a substructure in the 30 kD region of the gel, suggesting heterogeneity due to varying degrees of glycosylation. After reduction (FIGURE 4B), Con A blots show evidence for two major components at 16 kD and 18 kD. In addition, it has been demonstrated that no glycosylated material remains at the 30 kD region after reduction.

[0060] In order to confirm the presence of carbohydrate and to estimate the amount of carbohydrate attached, the 30 kD protein is treated' with N-glycanase, a deglycosylating enzyme with a broad specificity. Samples of the ^{125}I-labelled 30 kD protein are incubated with the enzyme in the presence of SDS for 24 hours at 37°C. As observed by SDS-PAGE, the treated samples appear as a prominent species at about 27 kD (FIGURE 5A). Upon reduction, the 27 kD species is reduced to species having a molecular weight of about 14 kD - 16 kD (FIGURE 5B).

[0061] To ensure complete deglycosylation of the 30KD protein, chemical cleavage of the carbohydrate moieties using hydrogen fluoride (HF) is performed. Active osteogenic protein fractions pooled from the C-18 chromatography step are dried in vacuo over P_2O_5 in a polypropylene tube, and 50 µl freshly distilled anhydrous HF at -70°C is added. After capping the tube tightly, the mixture is kept at 0°C in an ice-bath with occasional agitation for 1 hr. The HF is then evaporated using a continuous stream of dry nitrogen gas. The tube is removed from the ice bath and the residue dried in vacuo over P_2O_5 and KOH pellets.

[0062] Following drying, the samples are dissolved in 100 µl of 50% acetonitrile/0.1% TFA and aliquoted for SDS gel analysis, Con A binding, and biological assay. Aliquots are dried and dissolved in either SDS gel sample buffer in preparation for SDS gel analysis and Con A blotting or 4 M guanidine-HCl, 50 mM Tris-HCl, pH 7.0 for biological assay.

[0063] The results show that samples are completely deglycosylated by the HF treatment: Con A blots after SDS gel electrophoreses and transfer to Immobilon membrane showed no binding of Con A to the treated samples, while untreated controls were strongly positive at 30 kD. Coomassie gels of treated samples showed the presense of a 27 kD band instead of the 30 kD band present in the untreated controls.

C3. Chemical and Enzymatic Cleavage

[0064] Cleavage reactions with CNBr are analyzed using Con A binding for detection of fragments associated with carbohydrate. Cleavage reactions are conducted using trifluoroacetic acid (TFA) in the presence and absence of CNBr. Reactions are conducted at 37°C for 18 hours, and the samples are vacuum dried. The samples are washed with water, dissolved in SDS gel sample buffer with reducing agent, boiled and applied to an SDS gel. After electrophoresis, the protein is transferred to Immobilon membrane and visualized by Con A binding. In low concentrations of acid (1%), CNBr cleaves the majority of 16 kD and 18 kD species to one product, a species about 14 kD. In reactions using 10% TFA, a 14 kD species is observed both with and without CNBr.

[0065] Four proteolytic enzymes are used in these experiments to examine the digestion products of the 30 kD protein: 1) V-8 protease; 2) Endo Lys C protease; 3) pepsin; and 4) trypsin. Except for pepsin, the digestion buffer for the enzymes is 0.1 M ammonium bicarbonate, pH 8.3. The pepsin reactions are done in 0.1% TFA. The digestion volume is 100 µl and the ratio of enzyme to substrate is 1:10. ^{125}I-labelled 30 kD osteogenic protein is added for detection. After incubation at 37°C for 16 hr., digestion mixtures are dried down and taken up in gel sample buffer containing dithiothreitol for SDS-PAGE. FIGURE 6 shows an autoradiograph of an SDS gel of the digestion products. The results show that under these conditions, only trypsin digests the reduced 16 kD/18 kD species completely and yields a major species at around 12 kD. Pepsin digestion yields better defined, lower molecular weight species. However, the 16 kD/18 kD fragments were not digested completely. The V-8 digest shows limited digestion with one dominant species at 16 kD.

C4. Protein Sequencing

[0066] To obtain amino acid sequence data, the protein is cleaved with trypsin or Endoproteinase Asp-N (EndoAsp-N). The tryptic digest of reduced and carboxymethylated 30 kD protein (approximately 10 µg) is fractionated by reverse-phase HPLC using a C-8 narrowbore column (13 cm x 2.1 mm ID) with a TFA/acetonitrile gradient and a flow rate of 150 µl/min. The gradient employs (A) 0.06% TFA in water and (B) 0.04% TFA in water and acetonitrile (1:4; v:v). The procedure was 10% B for five min., followed by a linear gradient for 70 min. to 80% B, followed by a linear gradient for 10 min. to 100% B. Fractions containing fragments as determined from the peaks in the HPLC profile (FIGURE 7A) are rechromatographed at least once under the same conditions in order to isolate single components satisfactory for sequence analysis.

[0067] The HPLC profiles of the similarly digested 16 kD and 18 kD subunits are shown in FIGURES 7B and 7C,

respectively. These peptide maps are similar suggesting that the subunits are identical or are closely related.

[0068] The 16 kD and 18 kD subunits are digested with EndoAsp-N proteinase. The protein is treated with 0.5 µg EndoAsp-N in 50 mM sodium phosphate buffer, pH 7.8 at 36°C for 20 hr. The conditions for fractionation are the same as those described previously for the 30 kD, 16 kD, and 18 kD digests. The profiles obtained are shown in FIGURES 16A and 16B.

[0069] Various peptide fragments produced using the foregoing procedures have been analyzed in an automated amino acid sequencer (Applied Biosystems 470A with 120A on-line PTH analysis). The following sequence data has been obtained:

(1) S-F-D-A-Y-Y-C-S-G-A-C-Q-F-P-M-P-K;

(2) S-L-K-P-S-N-Y-A-T-I-Q-S-I-V;

(3) A-C-C-V-P-T-E-L-S-A-I-S-M-L-Y-L-D-E-N-E-K;

(4) M-S-S-L-S-I-L-F-F-D-E-N-K;

(5) S-Q-E-L-Y-V-D-F-Q-R;

(6) F-L-H-C-Q-F-S-E-R-N-S;

(7) T-V-G-Q-L-N-E-Q-S-S-E-P-N-I-Y;

(8) L-Y-D-P-M-V-V;

(9) V-G-V-V-P-G-I-P-E-P-C-C-V-P-E;

(10) V-D-F-A-D-I-G;

(11) V-P-K-P-C-C-A-P-T;

(12) I-N-I-A-N-Y-L;

(13) D-N-H-V-L-T-M-F-P-I-A-I-N;

(14) D-E-Q-T-L-K-K-A-R-R-K-Q-W-I-?-P;

(15) D-I-G-?-S-E-W-I-I-?-P;

(16) S-I-V-R-A-V-G-V-P-G-I-P-E-P-?-?-V;

(17) D-?-I-V-A-P-P-Q-Y-H-A-F-Y;

(18) D-E-N-K-N-V-V-L-K-V-Y-P-N-M-T-V-E;

(19) S-Q-T-L-Q-F-D-E-Q-T-L-K-?-A-R-?-K-Q;

(20) D-E-Q-T-L-K-K-A-R-R-K-Q-W-I-E-P-R-N-?-A-R-R-Y-L;

(21) A-R-R-K-Q-W-I-E-P-R-N-?-A-?-R-Y-?-?-V-D; and

(22) R-?-Q-W-I-E-P-?-N-?-A-?-?-Y-L-K-V-D-?-A-?-?-G

C5. Amino Acid Analysis

[0070] Samples of oxidized (30 kD) and reduced (16 kD and 18 kD) BOP are electrophoresed on a gel and transferred to Immobilon for hydrolysis and amino acid analysis using conventional, commercially available reagents to derivatize samples and HPLC using the PicO Tag (Millipore) system. The composition data generated by amino acid analyses of 30 kD BOP is reproducible, with some variation in the number of residues for a few amino acids, especially cysteine and isoleucine.

[0071] Composition data obtained are shown in TABLE 5.

TABLE 5

BOP Amino Acid Analyses			
Amino Acid	30 kD	16 kD	18 kD
Aspartic Acid/ Asparagine	22	14	15
Glutamic Acid/ Glutamine	24	14	16
Serine	24	16	23
Glycine	29	18	26
Histidine	5	*	4
Arginine	13	6	6
Threonine	11	6	7
Alanine	18	11	12
Proline	14	6	6
Tyrosine	11	3	3
Valine	14	8	7
Methionine	3	0	2
Cysteine**	16	14	12
Isoleucine	15	14	10
Leucine	15	8	9
Phenylalanine	7	4	4
Tryptophan	ND	ND	ND
Lysine	12	6	6

*This result is not integrated because histidine is present in low quantities.

**Cysteine is corrected by percent normally recovered from performic acid hydrolysis of the standard protein.

[0072] The results obtained from the 16 kD and 18 kD subunits, when combined, closely resemble the numbers obtained from the native 30 kD protein. The high figures obtained for glycine and serine are most likely the result of gel elution.

D. PURIFICATION OF HUMAN OSTEOGENIC PROTEIN

[0073] Human bone is obtained from the Bone Bank, (Massachusetts General Hospital, Boston, MA), and is milled, defatted, demarrowed and demineralized by the procedure disclosed above. 320 g of mineralized bone matrix yields 70 - 80 g of demineralized bone matrix. Dissociative extraction and ethanol precipitation of the matrix gives 12.5 g of guanidine-HCl extract.

[0074] One third of the ethanol precipitate (0.5 g) is used for gel filtration through 4 M guanidine-HCl (FIGURE 10A). Approximately 70-80 g of ethanol precipitate per run is used. In vivo bone inducing activity is localized in the fractions containing proteins in the 30 kD range. They are pooled and equilibrated in 6 M urea, 0.5 M NaCl buffer, and applied directly onto a HAP column; the bound protein is eluted stepwise by using the same buffer containing 100 mM and 500 mM phosphate (FIGURE 10B). Bioassay of HAP bound and unbound fractions demonstrates that only the fraction eluted by 100 mM phosphate has bone inducing activity in vivo. The biologically active fraction obtained from HAP chromatography is subjected to heparin-Sepharose affinity chromatography in buffer containing low salt; the bound proteins are eluted by 0.5 M NaCl (FIGURE 10C). Assaying the heparin-Sepharose fractions shows that the bound fraction eluted by 0:5 M NaCl have bone-inducing activity. The active fraction is then subjected to C-18 reverse phase chromatography. (FIGURE 10D).

[0075] The active fraction can then be subjected to SDS-PAGE as noted above to yield a band at about 30 kD comprising substantially pure human osteogenic protein.

E. BIOSYNTHETIC PROBES FOR ISOLATION OF GENES ENCODING NATIVE OSTEOGENIC PROTEIN

E-1 PROBE DESIGN

[0076] A synthetic consensus gene shown in FIGURE 13 was designed as a hybridization probe based on amino acid predictions from homology with the TGF-beta gene family and using human codon bias as found in human TGF-

beta. The designed concensus sequence was then constructed using known techniques involving assembly of oligonucleotides manufactured in a DNA synthesizer.

[0077] Tryptic peptides derived from BOP and sequenced by Edman degradation provided amino acid sequences that showed strong homology with the Drosophila DPP protein sequence (as inferred from the gene), the Xenopus VG1 protein, and somewhat less homology to inhibin and TGF-beta, as demonstrated below in TABLE 6.

TABLE 6

protein	amino acid sequence	homology
(BOP)	SFDAYYCSGACQFPS ***** * * **	(9/15 matches)
(DPP)	GYDAYYCHGKCPFFL	
(BOP)	SFDAYYCSGACQFPS * ** * * *	(6/15 matches)
(Vg1)	GYMANYCYGECPYPL	
(BOP)	SFDAYYCSGACQFPS * ** * *	(5/15 matches)
(inhibin)	GYHANYCEGECPSHI	
(BOP)	SFDAYYCSGACQFPS * * * *	(4/15 matches)
(TGF-beta)	GYHANFCLGPCPYIW	
(BOP)	K/RACCVPTELSAISMLYLDEN ***** * **** * *	(12/20 matches)
(Vg1)	LPCCVPTKMSPISMLFYDNN	

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(BOP)        K/RACCVPTELSAISMLYLDEN
                * *****  *    **** *    (12/20 matches)
(inhibin)      KSCCVPTKLRPMSMLYYDDG

-------------------------------------------------------

(BOP)        K/RACCVPTELSAISMLYLDE
                 ****  *      *        (6/19 matches)
(TGF-beta)     APCCVPQALEPLPIVYYVG

-------------------------------------------------------

(BOP)        K/RACCVPTELSAISMLYLDEN
                *******  *     ****    (12/20 matches)
(DPP)          KACCVPTQLDSVAMLYLNDQ

-------------------------------------------------------

(BOP)        LYVDF
             *****                      (5/5 matches)
(DPP)        LYVDF

-------------------------------------------------------

(BOP)        LYVDF
             *** *                      (4/5 matches)
(Vgl)        LYVEF

-------------------------------------------------------

(BOP)        LYVDF
             ** **                      (4/5 matches)
(TGF-beta)   LYIDF

-------------------------------------------------------

(BOP)        LYVDF
               * *                      (2/5 matches)
(inhibin)    FFVSF

-------------------------------------------------------
```

*-match

[0078] In determining the amino acid sequence of an osteogenic protein (from which the nucleic acid sequence can be determined), the following points were considered: (1) the amino acid sequence determined by Edman degradation of osteogenic protein tryptic fragments is ranked highest as long as it has a strong signal and shows homology or conservative changes when aligned with the other members of the gene family; (2) where the sequence matches for all four proteins, it is used in the synthetic gene sequence; (3) matching amino acids in DPP and Vgl are used; (4) If Vgl or DPP diverged but either one were matched by inhibin or by TGF-beta, this matched amino acid is chosen; (5) where all sequences diverged, the DPP sequence is initially chosen, with a later plan of creating the Vgl sequence by mutagenesis kept as a possibility. In addition, the consensus sequence is designed to preserve the disulfide crosslinking

and the apparent structural homology.

[0079] One purpose of the originally designed synthetic consensus gene sequence, designated COP0, (see FIGURE 13), was to serve as a probe to isolate natural genes. For this reason the DNA was designed using human codon bias. Alternatively, probes may be constructed using conventional techniques comprising a group of sequences of nucleotides which encode any portion of the amino acid sequence of the osteogenic protein produced in accordance with the foregoing isolation procedure. Use of such pools of probes also will enable isolation of a DNA encoding the intact protein.

E-2 Retrieval of Genes Encoding Osteogenic Protein from Genomic Library

[0080] A human genomic library (Maniatis-library) carried in lambda phage (Charon 4A) was screened using the COP0 consensus gene as probe. The initial screening was of 500,000 plaques (10 plates of 50,000 each). Areas giving hybridization signal were punched out from the plates, phage particles were eluted and plated again at a density of 2000-3000 plaques per plate. A second hybridization yielded plaques which were plated once more, this time at a density of ca 100 plaques per plate allowing isolation of pure clones. The probe (COP0) is a 300 base pair BamHI-PstI fragment restricted from an amplification plasmid which was labeled using alpha 32 dCTP according to the random priming method of Feinberg and Vogelstein (1984) Anal. Biochem. 137: 266-267. Prehybridization was done for 1 hr in 5x SSPE, 10x Denhardt's mix, 0.5% SDS at 50°C. Hybridization was overnight in the same solution as above plus probe. The washing of nitrocellulose membranes was done, once cold for 5 min. in 1x SSPE with 0.1% SDS and twice at 50°C for 2 x 30 min. in the same solution. Using this procedure, twenty-four positive clones were found. Two contained a gene never before reported designated OP1, osteogenic protein-1 described below. Two others yielded the genes corresponding to BMP-2b, one yielded BMP-3 (see PCT US 87/01537).

[0081] Southern blot analysis of lambda #13 DNA showed that an approximately 3kb BamHI fragment hybridized to the probe. (See FIGURE 1B). This fragment was isolated and subcloned into a bluescript vector (at the BamHI site). The clone was further analyzed by Southern blotting and hybridization to the COP0 probe. This showed that a 1 kb (approx.) EcoRI fragment strongly hybridized to the probe. This fragment was subcloned into the EcoRI site of a bluescript vector, and sequenced. Analysis of this sequence showed that the fragment encoded the carboxy terminus of a protein, named osteogenic protein-1 (OP1). The protein was identified by amino acid homology with the TGF-beta family. For this comparison cysteine patterns were used and then the adjacent amino acids were compared. Consensus splice signals were found where amino acid homologies ended, designating exon intron boundaries. Three exons were combined to obtain a functional TGF-beta-like domain containing seven cysteines. Two introns were deleted by looping out via primers bridging the exons using the single stranded mutagenesis method of Kunkel. Also, upstream of the first cysteine, an EcoRI site and an asp-pro junction for acid cleavage were introduced, and at the 3' end a PstI site was added by the same technique. Further sequence information (penultimate exon) was obtained by sequencing the entire insert. The sequencing was done by generating a set of unidirectionally deleted clones (Ozkaynak, E., and Putney, S. (1987) Biotechniques, 5:770-773). The obtained sequence covers about 80% of the TGF-beta-like region of OP1 and is set forth in FIGURE 1A. The complete sequence of the TGF-beta like region was obtained by first subcloning all EcoRI generated fragments of lambda clone #13 DNA and sequencing a 4 kb fragment that includes the first portion of the TGF-beta like region (third exon counting from end) as well as sequences characterized earlier. The gene on an EcoRI to PstI fragment was inserted into an E. coli expression vector controlled by the trp promoter-operator to produce a modified trp LE fusion protein with an acid cleavage site. The OP1 gene encodes amino acids corresponding substantially to a peptide found in sequences of naturally sourced material. The amino acid sequence of what is believed to be its active region is set forth below:

```
                1         10         20         30         40
OP1             LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                          50         60         70
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
     80         90        100
ISVLYFDDSSNVILKKYRNMVVRACGCH
```

A longer active sequence is:

```
                                                          -5
                                                          HQRQA
                  1        10         20         30         40
OP1               CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                                50         60         70
                  YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
                      80         90        100
                  ISVLYFDDSSNVILKKYRNMVVRACGCH
```

[0082] The amino acid sequence of what is believed to be the active regions encoded by the other three native genes retrieved using the consensus probe are:

```
                  1        10         20         30         40
CBMP-2a           CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
                                50         60         70
                  HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
                      80         90        100
                  ISMLYLDENEKVVLKNYQDMVVEGCGCR

                  1        10         20         30         40
CBMP-2b           CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
                                50         60         70
                  HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
                      80         90        100
                  ISMLYLDEYDKVVLKNYQEMVVEGCGCR

                  1        10         20         30         40
CBMP-3            CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPK
                                50         60         70
                  SLKPSN--H-ATIQSIVRAVGVVPGIPEPCCVPEKMSS
                      80         90        100
                  LSILFFDENKNVVLKVYPNMTVESCACR
```

E-3 Probing cDNA Library

[0083] Another example of the use of pools of probes to enable isolation of a DNA encoding the intact protein is shown by the following. Cells known to express the protein (e.g., osteoblasts or osteosarcoma) are extracted to isolate total cytoplasmic RNA. An oligo-dT column can be used to isolate mRNA. This mRNA can be size fractionated by, for example, gel electrophoresis. The fraction which includes the mRNA of interest may be determined by inducing transient expression in a suitable host cell and testing for the presence of osteogenic protein using, for example, antibody raised against peptides derived from the tryptic fragments of osteogenic protein in an immunoassay. The mRNA fraction is then reverse transcribed to single stranded cDNA using reverse transcriptase; a second complementary DNA strand can then be synthesized using the cDNA as a template. The double-standard DNA is then ligated into vectors which are used to transfect bacteria to produce a cDNA library.

[0084] The radiolabelled consensus sequence, portions thereof, and/or synthetic deoxy oligonucleotides complementary to codons for the known amino acid sequences in the osteogenic protein may be used to identify which of the DNAs in the cDNA library encode the full length osteogenic protein by standard DNA-DNA hybridization techniques.

[0085] The cDNA may then be integrated in an expression vector and transfected into an appropriate host cell for protein expression. The host may be a prokaryotic or eucaryotic cell since the former's inability to glycosylate osteogenic protein will not effect the protein's enzymatic activity. Useful host cells include Saccharomyces, E. coli, and various mammalian cell cultures. The vector may additionally encode various signal sequences for protein secretion and/or

may encode osteogenic protein as a fusion protein. After being translated, protein may be purified from the cells or recovered from the culture medium.

E4. Gene Preparation

[0086] Natural gene sequences and cDNAs retrieved as described above may be used for expression. The genes above may also be produced by assembly of chemically synthesized oligonucleotides. 15-100mer oligonucleotides may be synthesized on a Biosearch DNA Model 8600 Synthesizer, and purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer (TBE). The DNA is then electroeluted from the gel. Overlapping oligomers may be phosphorylated by T4 polynucleotide kinase and ligated into larger blocks which may also be purifed by PAGE.

E5. Expression

[0087] The genes can be expressed in appropriate prokaryotic hosts such as various strains of E. coli, and also in bacillus, yeasts, and various animal cells such as CHO, myeloma, etc. Generally, expression may be achieved using many cell types and expression systems well known to those skilled in the art. For example, if the gene is to be expressed in E. coli, an expression vector based on pBR322 and containing a synthetic trp promoter operator and the modified trp LE leader may be used. The vector can be opened at the EcoRI and PSTI restriction sites, and, for example, an OP gene fragment can be inserted between these two sites. The OP protein is joined to the leader protein via a hinge region having the sequence Asp-Pro. This hinge permits chemical cleavage of the fusion protein with dilute acid at the Asp-Pro site.

E6. Production of Active Proteins

[0088] The following procedure may be followed for production of active recombinant proteins. E. coli cells containing the fusion proteins are lysed. The fusion proteins are purified by differential solubilization. Cleavage is conducted with dilute acid, and the resulting cleavage products are passed through a Sephacryl-200HR or SP Trisacyl column to separate the cleaved proteins. The reduced OP fractions are then subjected to HPLC on a semi-prep C-18 column.

[0089] Conditions for refolding of OP were at pH 8.0 using 50 mM Tris-HCl and 6M Gu-HCl. Samples were refolded for 18 hours at 4°C.

[0090] These procedures have been used to express in E. coli on the active protein designated OPI having the amino acid sequence set forth above (longer species).

[0091] Refolding may not be required if the proteins are expressed in animal cells.

MATRIX PREPARATION

A. General Consideration of Matrix Properties

[0092] The carrier described in the bioassay section, infra, may be replaced by either a biodegradable-synthetic or synthetic-inorganic matrix (e.g., HAP, collagen, tricalcium phosphate, or polylactic acid, polyglycolic acid and various copolymers thereof). Also xenogeneic bone may be used if pretreated as described below.

[0093] Studies have shown that surface charge, particle size, the presence of mineral, and the methodology for combining matrix and osteogenic protein all play a role in achieving successful bone induction. Perturbation of the charge by chemical modification abolishes the inductive response. Particle size influences the quantitative response of new bone; particles between 75 and 420 μm elicit the maximum response. Contamination of the matrix with bone mineral will inhibit bone formation. Most importantly, the procedures used to formulate osteogenic protein onto the matrix are extremely sensitive to the physical and chemical state of both the osteogenic protein and the matrix.

[0094] The sequential cellular reactions at the interface of the bone matrix/OP implants are complex. The multistep cascade includes: binding of fibrin and fibronectin to implanted matrix, chemotaxis of cells, proliferation of fibroblasts, differentiation into chondroblasts, cartilage formation, vascular invasion, bone formation, remodeling, and bone marrow differentiation.

[0095] A successful carrier for osteogenic protein must perform several important functions. It must bind osteogenic protein and act as a slow release delivery system, accommodate each step of the cellular response during bone development, and protect the osteogenic protein from nonspecific proteolysis. In addition, selected materials must be biocompatible in vivo and biodegradable; the carrier must act as a temporary scaffold until replaced completely by new bone. Biocompatibility requires that the matrix not induce significant inflammation when implanted and not be rejected by the host animal. Biodegradability requires that the matrix be slowly absorbed by the body of the host during development of new bone or cartilage. Polylactic acid (PLA), polyglycolic acid (PGA), and various combinations have different

dissolution rates in vivo. In bones, the dissolution rates can vary according to whether the implant is placed in cortical or trabecular bone.

[0096] Matrix geometry, particle size, the presence of surface charge, and porosity or the presence of interstices among the particles of a size sufficient to permit cell infiltration, are all important to successful matrix performance. It is preferred to shape the matrix to the desired form of the new bone and to have dimensions which span non-union defects. Rat studies show that the new bone is formed essentially having the dimensions of the device implanted.

[0097] The matrix may comprise a shape-retaining solid made of loosely adhered particulate material, e.g., with collagen. It may also comprise a molded, porous solid, or simply an aggregation of close-packed particles held in place by surrounding tissue. Masticated muscle or other tissue may also be used. Large allogeneic bone implants can act as a carrier for the matrix if their marrow cavities are cleaned and packed with particles and the dispersed osteogenic protein.

B. Preparation of Biologically Active Allogenic Matrix

[0098] Demineralized bone matrix is prepared from the dehydrated diaphyseal shafts of rat femur and tibia as described herein to produce a bone particle size which pass through a 420 μ sieve. The bone particles are subjected to dissociative extraction with 4 M guanidine-HCl. Such treatment results in a complete loss of the inherent ability of the bone matrix to induce endochondral bone differentiation. The remaining insoluble material is used to fabricate the matrix. The material is mostly collagenous in nature, and upon implantation, does not induce cartilage and bone. All new preparations are tested for mineral content and false positives before use. The total loss of biological activity of bone matrix is restored when an active osteoinductive protein fraction or a pure protein is reconstituted with the biologically inactive insoluble collagenous matrix. The osteoinductive protein can be obtained from any vertebrate, e.g., bovine, porcine, monkey, or human, or produced using recombinant DNA techniques.

C. Preparation of Deglycosylated Bone Matrix for Use in Xenogenic Implant

[0099] When osteogenic protein is reconstituted with collagenous bone matrix from other species and implanted in rat, no bone is formed. This suggests that while the osteogenic protein is xenogenic (not species specific), the matrix is species specific and cannot be implanted cross species perhaps due to intrinsic immunogenic or inhibitory components. Thus, heretofore, for bone-based matrices, in order for the osteogenic protein to exhibit its full bone inducing activity, a species specific collagenous bone matrix was required.

[0100] The major component of all bone matrices is Type I collagen. In addition to collagen, extracted bone includes non-collagenous proteins which may account for 5% of its mass. Many non-collagenous components of bone matrix are glycoproteins. Although the biological significance of the glycoproteins in bone formation is not known, they may present themselves as potent antigens by virtue of their carbohydrate content and may constitute immunogenic and/or inhibitory components that are present in xenogenic matrix.

[0101] It has now been discovered that a collagenous bone matrix may be used as a carrier to effect bone inducing activity in xenogenic implants, if one first removes the immonogenic and inhibitory components from the matrix. The matrix is deglycosglated chemically using, for example, hydrogen fluoride to achieve this purpose.

[0102] Bovine bone residue prepared as described above is sieved, and particles of the 74-420 μM are collected. The sample is dried in vacuo over P_2O_5, transferred to the reaction vessel and anhydrous hydrogen fluoride (HF) (10-20 ml/g of matrix) is then distilled onto the sample at -70°C. The vessel is allowed to warm to 0°C and the reaction mixture is stirred at this temperature for 120 min. After evaporation of the HF in vacuo, the residue is dried thoroughly in vacuo over KOH pellets to remove any remaining traces of acid.

[0103] Extent of deglycosylation can be determined from carbohydrate analysis of matrix samples taken before and after treatment with HF, after washing the samples appropriately to remove non-covalently bound carbohydrates.

[0104] The deglycosylated bone matrix is next treated as set forth below:

1) suspend in TBS (Tris-buffered Saline) 1g/200 ml and stir at 4°C for 2 hrs or in 6 M urea, 50 mM Tris-HCl, 500 mM NaCl, pH 7.0 (UTBS), and stir at RT for 30 min.;

2) centrifuge and wash with TBS or UTBS as in step 1); and

3) centrifuge; discard supernatant; water wash residue; and then lyophilize.

FABRICATION OF OSTEOGENIC DEVICE

[0105] Fabrication of osteogenic devices using any of the matrices set forth above with any of the osteogenic proteins

described above may be performed as follows.

A. Ethanol precipitation

[0106] In this procedure, matrix was added to osteogenic protein in guanidine-HCl. Samples were vortexed and incubated at a low temperature. Samples were then further vortexed. Cold absolute ethanol was added to the mixture which was then stirred and incubated. After centrifugation (microfuge high speed) the supernatant was discarded. The reconstituted matrix was washed with cold concentrated ethanol in water and then lyophilized.

B. Acetonitrile Trifluoroacetic Acid Lyophilization

[0107] In this procedure, osteogenic protein in an acetonitrile trifluroacetic acid (ACN/TFA) solution was added to the carrier. Samples were vigorously vortexed many times and then lyophilized.

C. Urea Lyophilization

[0108] For those proteins that are prepared in urea buffer, the protein is mixed with the matrix, vortexed many times, and then lyophilized. The lyophilized material may be used "as is" for implants.

IN VIVO RAT BIOASSAY

[0109] Substantially pure BOP, BOP-rich extracts comprising protein having the properties set forth above, and several of the recombinant proteins have been incorporated in matrices to produce osteogenic devices, and assayed in rat for endochondral bone. Studies in rats show the osteogenic effect to be dependent on the dose of osteogenic protein dispersed in the osteogenic device. No activity is observed if the matrix is implanted alone. The following sets forth guidelines for how the osteogenic devices disclosed herein might be assayed for determining active fractions of osteogenic protein when employing the isolation procedure of the invention, and evaluating protein constructs and matrices for biological activity.

A. Subcutaneous Implantation

[0110] The bioassay for bone induction as described by Sampath and Reddi (Proc. Natl. Acad. Sci. USA (1983) 80: 6591-6595), herein incorporated by reference, is used to monitor the purification protocols for endochondral bone differentiation activity. This assay consists of implanting the test samples in subcutaneous sites in allogeneic recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, were used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoraic region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day of the experiment. Implants were removed on day 12. The heterotopic site allows for the study of bone induction without the possible ambiguities resulting from the use of orthotopic sites.

B. Cellular Events

[0111] The implant model in rats exhibits a controlled progression through the stages of matrix induced endochondral bone development including: (1) transient infiltration by polymorphonuclear leukocytes on day one; (2) mesenchymal cell migration and proliferation on days two and three; (3) chondrocyte appearance on days five and six; (4) cartilage matrix formation on day seven; (5) cartiliage calcification on day eight; (6) vascular invasion, appearance of osteoblasts, and formation of new bone on days nine and ten; (7) appearance of osteoblastic and bone remodeling and dissolution of the implanted matrix on days twelve to eighteen; and (8) hematopoietic bone marrow differentiation in the ossicle on day twenty-one. The results show that the shape of the new bone conforms to the shape of the implanted matrix.

C. Histological Evaluation

[0112] Histological sectioning and staining is preferred to determine the extent of osteogenesis in the implants. Implants are fixed in Bouins Solution, embedded in parafilm, cut into 6-8 mm sections. Staining with toluidine blue or hemotoxylin/eosin demonstrates clearly the ultimate development of endochondrial bone. Twelve day implants are usually sufficient to determine whether the implants show bone inducing activity.

D. Biological Markers

[0113] Alkaline phosphatase activity may be used as a marker for osteogenesis. The enzyme activity may be determined spectrophotometrically after homogenization of the implant. The activity peaks at 9-10 days in vivo and thereafter slowly declines. Implants showing no bone development by histology should have little or no alkaline phosphatase activity under these assay conditions. The assay is useful for quantitation and obtaining an estimate of bone formation very quickly after the implants are removed from the rat. Alternatively the amount of bone formation can be determined by measuring the calcium content of the implant.

[0114] Implants containing osteogenic protein at several levels of purity have been tested to determine the most effective dose/purity level, in order to seek a formulation which could be produced on a commercial scale. The results are measured by specific acivity of alkaline phosphatase, calcium content, and histological examination. As noted previously, the specific activity of alkaline phosphatase is elevated during onset of bone formation and then declines. On the other hand, calcium content is directly proportional to the total amount of bone that is formed. The osteogenic activity due to osteogenic protein is represented by "bone forming units". For example, one bone forming unit represents the amount of protein that is needed for half maximal bone forming activity as compared to rat demineralized bone matrix as control and determined by calcium content of the implant on day 12.

E. Results

[0115] Dose curves are constructed for bone inducing activity in vivo at each step of the purification scheme by assaying various concentrations of protein. FIGURE 11 shows representative dose curves in rats as determined by alkaline phosphatase. Similar results are obtained when represented as bone forming units. Approximately 10-12 μg of the TSK-fraction, 3-4 μg of heparin-Sepharose-II fraction, 0.4-0.5 μg of the C-18 column purified fraction, and 20-25 ng of gel eluted highly purified 30 kD protein is needed for unequivocal bone formation (half maximum activity). 20-25 ng of the substantially pure protein per 25 mg of implant is normally sufficient to produce endochondral bone. Thus, 1-2 ng osteogenic protein per mg of implant is a reasonable dosage, although higher dosages may be used. (See section IB5 on specific activity of osteogenic protein.)

[0116] OP1 expressed as set forth above (longer version), when assayed for activity histologically, induced cartilage and bone formation as evidenced by the presence of numerous chondrocytes in many areas of the implant and by the presence of osteoblasts surrounding vascular endothelium forming new matrix.

[0117] Deglycosylated xenogenic collagenous bone matrix (example: bovine) has been used instead of allogenic collagenous matrix to prepare osteogenic devices (see previous section) and bioassayed in rat for bone inducing activity in vivo. The results demonstrate that xenogenic collagenous bone matrix after chemical deglycosylation induces successful endochondral bone formation (FIGURE 19). As shown by specific activity of alkaline phosphotase, it is evident that the deglycosylated xenogenic matrix induced bone whereas untreated bovine matrix did not.

[0118] Histological evaluation of implants suggests that the deglycosylated bovine matrix not only has induced bone in a way comparable to the rat residue matrix but also has advanced the developmental stages that are involved in endochondral bone differentiation. Compared to rat residue as control, the HF treated bovine matrix contains extensively remodeled bone. Ossicles are formed that are already filled with bone marrow elements by 12 days. This profound action as elicited by deglycosylated bovine matrix in supporting bone induction is reproducible and is dose dependent with varying concentration of osteogenic protein.

ANIMAL EFFICACY STUDIES

[0119] Substantially pure osteogenic protein from bovine bone (BOP), BOP-rich osteogenic fractions having the properties set forth above, and several recombinant proteins have been incorporated in matrices to produce osteogenic devices. The efficacy of bone-inducing potential of these devices was tested in cat and rabbit models, and found to be potent inducers of osteogenesis, ultimately resulting in formation of mineralized bone. The following sets forth guidelines as to how the osteogenic devices disclosed herein might be used in a clinical setting.

A. Feline Model

[0120] The purpose of this study is to establish a large animal efficacy model for the testing of the osteogenic devices of the invention, and to characterize repair of massive bone defects and simulated fracture non-union encountered frequently in the practice of orthopedic surgery. The study is designed to evaluate whether implants of osteogenic protein with a carrier can enhance the regeneration of bone following injury and major reconstructive surgery by use of this large mammal model. The first step in this study design consists of the surgical preparation of a femoral osteotomy defect which, without further intervention, would consistently progress to non-union of the simulated fracture defect.

The effects of implants of osteogenic devices into the created bone defects were evaluated by the following study protocol.

A-1. Procedure

[0121] Sixteen adult cats weighing less than 10 lbs. undergo unilateral preparation of a 1 cm bone defect in the right femur through a lateral surgical approach. In other experiments, a 2 cm bone defect was created. The femur is immediately internally fixed by lateral placement of an 8-hole plate to preserve the exact dimensions of the defect. There are three different types of materials implanted in the surgically created feline femoral defects: group I (n = 3) is a control group which undergo the same plate fixation with implants of 4 M guanidine-HCl-treated (inactivated) feline demineralized bone matrix powder (Gu-HCl-DBM) (360 mg); group II (n = 3) is a positive control group implanted with biologically active feline demineralized bone matrix powder (DBM) (360 mg); and group III (n = 10) undergo a procedure identical to groups I-II, with the addition of osteogenic protein onto each of the Gu-HCl-DBM carrier samples. To summarize, the group III osteogenic protein-treated animals are implanted with exactly the same material as the group I animals, but with the singular addition of osteogenic protein.

[0122] All animals are allowed to ambulate ad libitum within their cages post-operatively. All cats are injected with tetracycline (25 mg/kg SQ each week for four weeks) for bone labelling. All but four group III animals are sacrificed four months after femoral osteotomy.

A-2. Radiomorphometrics

[0123] In vivo radiomorphometric studies are carried out immediately post-op at 4, 8, 12 and 16 weeks by taking a standardized x-ray of the lightly anesthesized animal positioned in a cushioned x-ray jig designed to consistently produce a true anterio-posterior view of the femur and the osteotomy site. All x-rays are taken in exactly the same fashion and in exactly the same position on each animal. Bone repair is calculated as a function of mineralization by means of random point analysis. A final specimen radiographic study of the excised bone is taken in two planes after sacrifice. X-ray results are shown in FIGURE 12, and displaced as percent of bone defect repair. To summarize, at 16 weeks, 60% of the group III femors are united with average 86% bone defect regeneration. By contrast, the group I Gu-HCl-DMB negative-control implants exhibit no bone growth at four weeks, less than 10% at eight and 12 weeks, and 16% (± 10%) at 16 weeks with one of the five exhibiting a small amount of bridging bone. The group II DMB positive-control implants exhibited 18% (± 3%) repair at four weeks, 35% at eight weeks, 50% (± 10%) at twelve weeks and 70% (± 12%) by 16 weeks, a statistical difference of $p < 0.01$ compared to osteogenic protein at every month. One of the three (33%) is united at 16 weeks.

A-3. Biomechanics

[0124] Excised test and normal femurs are immediately studied by bone densitometry, wrapped in two layers of saline-soaked towels, placed in two sealed plastic bags, and stored at -20° C until further study. Bone repair strength, load to failure, and work to failure are tested by loading to failure on a specially designed steel 4-point bending jig attached to an Instron testing machine to quantitate bone strength, stiffness, energy absorbed and deformation to failure. The study of test femurs and normal femurs yield the bone strength (load) in pounds and work to failure in joules. Normal femurs exhibit a strength of 96 (± 12) pounds. osteogenic protein-implanted femurs exhibited 35 (± 4) pounds, but when corrected for surface area at the site of fracture (due to the "hourglass" shape of the bone defect repair) this correlated closely with normal bone strength. Only one demineralized bone specimen was available for testing with a strength of 25 pounds, but, again, the strength correlated closely with normal bone when corrected for fracture surface area.

A-4. Histomorphometry/Histology

[0125] Following biomechanical testing the bones are immediately sliced into two longitudinal sections at the defect site, weighed, and the volume measured. One-half is fixed for standard calcified bone histomorphometrics with fluorescent stain incorporation evaluation, and one-half is fixed for decalcified hemotoxylin/eosin stain histology preparation.

A-5. Biochemistry

[0126] Selected specimens from the bone repair site (n=6) are homogenized in cold 0.15 M NaCl, 3 mM $NaHCO_3$, pH 9.0 by a Spex freezer mill. The alkaline phosphatase activity of the supernatant and total calcium content of the

acid soluble fraction of sediment are then determined.

A-6. Histopathology

[0127] The final autopsy reports reveal no unusual or pathologic findings noted at necropsy of any of the animals studied. Portion of all major organs are preserved for further study. A histopathological evaluation is performed on samples of the following organs: heart, lung, liver, both kidneys, spleen, both adrenals, lymph nodes, left and right quadriceps muscles at mid-femur (adjacent to defect site in experimental femur). No unusual or pathological lesions are seen in any of the tissues. Mild lesions seen in the quadriceps muscles are compatible with healing responses to the surgical manipulation at the defect site. Pulmonary edema is attributable to the euthanasia procedure. There is no evidence of any general systemic effects or any effects on the specific organs examined.

A-7. Feline Study Summary

[0128] The 1 cm and 2 cm femoral defect cat studies demonstrate that devices comprising a matrix containing disposed osteogenic protein can: (1) repair a weight-bearing bone defect in a large animal; (2) consistently induces bone formation shortly following (less than two weeks) implantation; and (3) induce bone by endochondral ossification, with a strength equal to normal bone, on a volume for volume basis. Furthermore, all animals remained healthy during the study and showed no evidence of clinical or histological laboratory reaction to the implanted device. In this bone defect model, there was little or no healing at control bone implant sites. The results provide evidence for the successful use of osteogenic devices to repair large, non-union bone defects.

B. Rabbit Model:

B1. Procedure and Results

[0129] The purpose of this study is to establish a model in which there is minimal or no bone growth in the control animals, so that when bone induction is tested, only a strongly inductive substance will yield a positive result. Defects of 1.5 cm are created in the ulnae of rabbits with implantation of osteogenic devices or no implant.

[0130] Eight mature (greater than 10 lbs) New Zealand White rabbits with epiphyseal closure documented by X-ray were studied. Of these eight animals (one animal each was sacrificed at one and two weeks), 11 ulnae defects are followed for the full course of the eight week study. In all cases (n = 7) following osteo-periosteal bone resection, the no implant animals establish no radiographic union by eight weeks. All no implant animals develop a thin "shell" of bone growing from surrounding bone present at four weeks and, to a slightly greater degree, by eight weeks. In all cases (n = 4), radiographic union with marked bone induction is established in the osteogenic protein-implanted animals by eight weeks. As opposed to the no implant repairs, this bone repair is in the site of the removed bone.

[0131] Radiomorphometric analysis reveal 90% osteogenic protein-implant bone repair and 18% no-implant bone repair at sacrifice at eight weeks. At autopsy, the osteogenic protein bone appears normal, while "no implant" bone sites have only a soft fibrous tissue with no evidence of cartilage or bone repair in the defect site.

B-2. Allograft Device

[0132] In another experiment, the marrow cavity of the 1.5 cm ulnar defect is packed with activated osteogenic protein rabbit bone powder and the bones are allografted in an intercalary fashion. The two control ulnae are not healed by eight weeks and reveal the classic "ivory" appearance. In distinct contrast, the osteogenic protein-treated implants "disappear" radiographically by four weeks with the start of remineralization by six to eight weeks. These allografts heal at each end with mild proliferative bone formation by eight weeks.

[0133] This type of device serves to accelerate allograph repair.

B-3. Summary

[0134] These studies of 1.5 cm osteo-periosteal defects in the ulnae of mature rabbits show that: (1) it is a suitable model for the study of bone growth; (2) "no implant" or Gu-HCl negative control implants yield a small amount of periosteal-type bone, but not medullary or cortical bone growth; (3) osteogenic protein-implanted rabbits exhibited proliferative bone growth in a fashion highly different from the control groups; (4) initial studies show that the bones exhibit 50% of normal bone strength (100% of normal correlated vol:vol) at only eight weeks after creation of the surgical defect; and (5) osteogenic protein-allograft studies reveal a marked effect upon both the allograft and bone healing.

Claims

1. A process for the production of active osteogenic protein in unglycosylated form, the protein comprising a TGF-beta-like domain containing seven cysteines, the process comprising the steps of:

   (a) providing genetic material encoding the osteogenic protein,
   (b) introducing the genetic material into a prokaryotic host cell;
   (c) expressing the genetic material in the prokaryotic host cell, and
   (d) refolding the expressed protein by oxidation to produce an active osteogenic protein comprising a pair of polypeptide chains bonded in the unreduced state to form a homo- or heterodimeric species having a conformation such that the pair of polypeptide chains is capable of inducing endochondral bone formation when disposed within a matrix and implanted in a mammal.

2. The process of claim 1 wherein the protein:

   (a) comprises the amino acid sequence **VPKPCCAPT**;
   (b) comprises the amino acid sequence

```
1         10          20          30          40
      LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
             50          60          70
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
       80          90          100
ISVLYFDDSSNVILKKYRNMVVRACGCH; or
```

   (c) comprises the amino acid sequence

```
                                                -5
                                               HQRQA
 1         10          20          30          40
CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
             50          60          70
 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
     80          90          100
 ISVLYFDDSSNVILKKYRNMVVRACGCH; or
```

   (d) comprises the amino acid sequence

```
1         10          20          30          40
CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
             50          60          70
HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
       80          90          100
 ISMLYDENEKVVLKNYQDMVVEGCGCR; or
```

   (e) comprises the amino acid sequence

```
1       10          20          30          40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
              50          60          70
 HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
     80          90          100
ISMLYLDEYDKVVLKNYQEMVVEGCGCR; or
```

(f) comprises the amino acid sequence

```
1       10          20          30          40
CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPK
              50          60          70
SLKPSN--H-ATIQSIVRAVGVVPGIPEPCCVPEKMSS
     80          90          100
LSILFFDENKNVVLKVYPNMTVESCACR; or
```

(g) comprises that sequence of amino acids encoded by a DNA sequence retrievable by hybridization with the probe of Figure 13.

3. The process of claim 1 or claim 2 wherein the protein:

(a) has an apparent molecular weight of about 30 kD in the glycosylated native form as determined by SDS-PAGE; or
(b) has an apparent molecular weight of about 27 kD in deglycosylated form as determined by SDS-PAGE; or
(c) comprises two polypeptide chains which have an apparent molecular weight of about 16 kD and 18 kD in glycosylated form;
(d) comprises two polypeptide chains which have an apparent molecular weight of about 14 kD to 16 kD in unglycosylated form.

4. The process of any one of the preceding claims wherein the host cell is an E. coli host cell.

5. A process for producing an osteogenic device comprising disposing the protein of any one of the preceding claims in a matrix.

6. The process of claim 5 wherein the device is for implantation in a mammal and comprises:

(a) a biocompatible, in vivo biodegradable matrix defining pores of a dimension sufficient to permit influx, proliferation and differentiation of migratory progenitor cells from the body of said mammal, and
(b) the protein disposed in said matrix and accessible to said cells.

7. The process of claim 6 wherein said matrix comprises close-packed particulate matter having a particle size within the range of 70 to 420 μm.

8. The process of any one of claims 5-7 wherein said matrix comprises: (a) allogenic bone, e.g. demineralized, protein extracted, particulate, allogenic bone, (b) demineralized, protein extracted, particulate, deglycosylated xenogenic bone (which is e.g. treated with a protease), (c) demineralized, protein extracted, particulate xenogenic bone treated with HF, (d) materials selected from collagen, hydroxyapatite, calcium phosphates (e.g. tricalcium phosphate) and polymers comprising glycolic acid and/or lactic acid monomers, (e) a shape-retaining solid of loosely adhered particulate material e.g. collagen, (f) a porous solid, (g) masticated tissue, e.g. muscle or (h) the marrow cavity of allogenic bone.

9. The process of any of claims 5-8 wherein the device is for use in therapy, e.g. for inducing local cartilage and/or endochondral or heterotopic bone formation in a mammal by implanting the device in a mammal at a locus acces-

sible to migratory progenitor cells for periodontal treatment, cartilage repair, accelerating allograft repair and for the treatment of osteoarthritis or to repair weight-bearing bone defects, correct non-union fractures, acquired or congenital craniofacial and other skeletal or dental anomalies.

10. The process of claim 9 wherein the device is for use in inducing shaped heterotopic bone, wherein the shape of the heterotopic bone conforms to that of the implanted device.

Patentansprüche

1. Verfahren zur Herstellung eines wirksamen osteogenen Proteins in unglykosylierter Form, wobei das Protein eine TGF-beta-artige Domäne umfasst, die sieben Cysteine enthält, wobei das Verfahren die folgenden Schritte umfasst:

(a) Bereitstellen eines genetischen Materials, das das osteogene Protein kodiert,
(b) Einführen des genetischen Materials in eine prokaryontische Wirtszelle;
(c) Exprimieren des genetischen Materials in der prokaryontischen Wirtszelle, und
(d) Neufalten des exprimierten Proteins durch Oxidation, um ein aktives osteogenes Protein zu erzeugen, das zwei Polypeptidketten umfasst, die im nicht-reduzierten Zustand gebunden sind, um eine homo- oder heterodimere Spezies mit einer solchen Konformation zu bilden, dass die zwei Polypeptidketten dazu in der Lage sind, eine Ersatzknochenbildung zu induzieren, wenn sie in einer Matrix angeordnet und einem Säugetier implantiert werden.

2. Verfahren nach Anspruch 1,
wobei das Protein:

(a) die Aminosäuresequenz **VPKPCCAPT**; umfasst;
(b) die Aminosäuresequenz

```
1        10         20         30         40
      LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                50         60         70
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
      80         90        100
ISVLYFDDSSNVILKKYRNMVVRACGCH;
```

umfasst; oder
(c) die Aminosäuresequenz

```
                                          -5
                                         HQRQA
 1        10         20         30         40
CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                50         60         70
 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
     80         90        100
 ISVLYFDDSSNVILKKYRNMVVRACGCH;
```

umfasst; oder
(d) die Aminosäuresequenz

```
1       10          20          30          40
CKREPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
                50          60          70
HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
      80          90          100
 ISMLYDENEKVVLKNYQDMVVEGCGCR;
```

umfasst; oder
(e) die Aminosäuresequenz

```
1       10          20          30          40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
                50          60          70
 HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
      80          90          100
ISMLYLDEYDKVVLKNYQEMVVEGCGCR;
```

umfasst; oder
(f) die Aminosäuresequenz

```
1       10          20          30          40
CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPK
                50          60          70
SLKPSN--E-ATIQSIVRAVGVVPGIPEPCCVPEKMSS
     80          90          100
LSILFFDENKNVVLKVYPNMTVESCACR;
```

umfasst; oder
(g) diejenige Sequenz von Aminosäuren umfasst, die durch eine DNA-Sequenz codiert werden, die durch Hybridisierung mit der Sonde von Fig. 13 auffindbar sind.

3. Verfahren nach Anspruch 1 oder Anspruch 2,
wobei das Protein:

(a) eine apparente relative Molekülmasse von ungefähr 30 kD in der glykosylierten nativen Form aufweist, wie durch SDS-PAGE bestimmt wird; oder
(b) eine apparente relative Molekülmasse von ungefähr 27 kD in der deglykosylierten Form aufweist, wie durch SDS-PAGE bestimmt wird; oder
(c) zwei Polypeptidketten umfasst, die eine apparente relative Molekülmasse von ungefähr 16 kD und 18 kD in glykosylierter Form aufweisen;
(d) zwei Polypeptidketten umfasst, die eine apparente relative Molekülmasse von ungefähr 14 kD bis 16 kD in unglykosylierter Form aufweisen.

4. Verfahren nach einem der vorhergehenden Ansprüche,
wobei die Wirtszelle eine E. coli Wirtszelle ist.

5. Verfahren zur Herstellung einer osteogenen Vorrichtung, das die Anordnung des Proteins nach einem der vorhergehenden Ansprüche in einer Matrix umfasst.

6. Verfahren nach Anspruch 5,
wobei die Vorrichtung zur Implantation in ein Säugetier vorgesehen ist und Folgendes umfasst:

(a) eine biokompatible, in vivo biologisch abbaubare Matrix, die Poren einer Abmessung definiert, die aus-

reicht, um Einstrom, Proliferation und Differentiation von migratorischen Vorläuferzellen aus dem Körper des Säugetiers zu ermöglichen, und
(b) das Protein in der Matrix angeordnet und für die Zellen zugänglich ist.

7. Verfahren nach Anspruch 6,
wobei die Matrix eng gepacktes teilchenförmiges Material mit einer Teilchengröße im Bereich von 70 bis 420 µm umfasst.

8. Verfahren nach einem der Ansprüche 5 bis 7,
wobei die Matrix Folgendes umfasst: (a) allogenen Knochen, beispielsweise demineralisierten, Protein-extrahierten, teilchenförmigen, allogenen Knochen, (b) demineralisierten, Protein-extrahierten, teilchenförmigen, deglykosylierten xenogenen Knochen (der beispielsweise mit einer Protease behandelt wird), (c) demineralisierten, Protein-extrahierten, teilchenförmigen xenogenen Knochen, der mit HF behandelt wurde, (d) Materialien, die aus Kollagen, Hydroxyapatit, Calciumphophaten (beispielsweise Tricalciumphosphat) und Polymeren ausgewählt sind, die Glycolsäure und/oder Milchsäuremonomere umfassen, (e) einen die Form behaltenden Feststoff von locker haftendem teilchenförmigen Material, beispielsweise Kollagen, (f) einen porösen Feststoff, (g) zerkleinertes Gewebe, beispielsweise Muskelgewebe, oder (h) die Markhöhle des allogenen Knochens.

9. Verfahren nach einem der Ansprüche 5 bis 8,
wobei die Vorrichtung zur Verwendung in der Therapie vorgesehen ist, beispielsweise zur Induktion einer lokalen Knorpel- und/oder Ersatzknochen- oder heterotopen Knochen-Bildung in einem Säugetier, indem die Vorrichtung einem Säugetier an einem Ort implantiert wird, der migratorischen Vorläuferzellen zugänglich ist, zur periodontalen Behandlung, Knorpelinstandsetzung, beschleunigten Allotransplantatinstandsetzung und zur Behandlung der Osteoarthritis oder zur Instandsetzung von Defekten von gewichtstragenden Knochen, Korrektur von nicht-heilenden Frakturen, erworbenen oder angeborenen kraniofacialen und anderen Skelett- oder Zahnanomalien.

10. Verfahren nach Anspruch 9,
wobei die Vorrichtung zur Verwendung zur Induktion eines geformten heterotopen Knochens verwendet wird, wobei die Form des heterotopen Knochens derjenigen der implantierten Vorrichtung entspricht.

Revendications

1. Procédé pour la production d'une protéine ostéogéniquement active sous une forme non glycosylée, la protéine comprenant un domaine de type TGF-bêta contenant sept cystéines, le procédé comprenant les étapes consistant à:

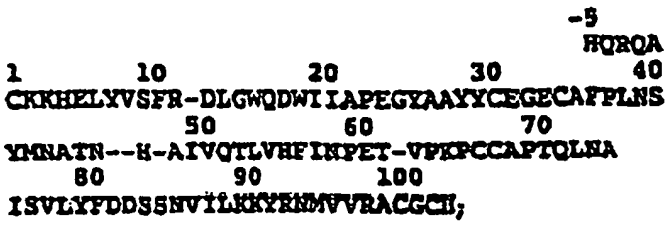
(a) fournir un matériau génétique codant la protéine ostéogène,
(b) introduire le matériau génétique dans une cellule-hôte procaryote,
(c) exprimer le matériau génétique dans la cellule-hôte procaryote, et
(d) replier par oxydation la protéine exprimée, pour produire une protéine ostéogéniquement active comprenant une paire de chaînes de polypeptides liées à l'état non réduit pour former une substance homo- ou hétérodimère dont la conformation est telle que la paire de chaînes de polypeptides est en mesure d'induire la formation d'os endochondrien lorsqu'elle est disposée dans une matrice et implantée chez un mammifère.

2. Procédé selon la revendication 1, dans lequel la protéine:

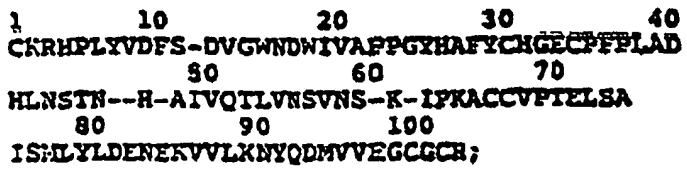
(a) comprend la séquence d'acides aminés VPKPCCAPT;
(b) comprend la séquence d'acides aminés

```
1         10         20         30         40
      LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
               50         60         70
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
     80         90        100
ISVLYFDDSSNVILKKYRNMVVRACGCH,
```

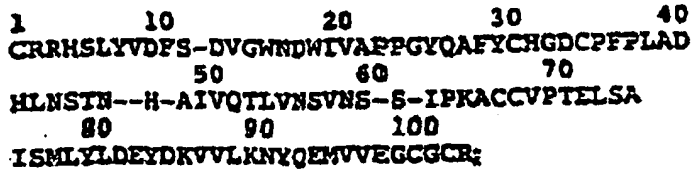
ou
(c) comprend la séquence d'acides aminés



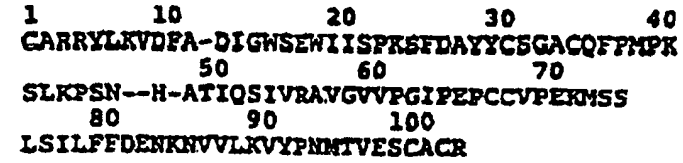
ou
(d) comprend la séquence d'acides aminés



ou
(e) comprend la séquence d'acides aminés



ou
(f) comprend la séquence d'acides aminés



ou
(g) comprend cette séquence d'acides aminés codée par une séquence d'ADN qui peut être récupérée par hybridation avec la sonde de la figure 13.

3. Procédé selon la revendication 1 ou la revendication 2, dans lequel la protéine:

(a) sous forme native glycosylée, présente une masse moléculaire apparente d'environ 30 kD telle que déterminée par SDS-PAGE;
(b) sous forme déglycosylée, présente une masse moléculaire apparente d'environ 27 kD telle que déterminée par SDS-PAGE; ou
(c) comprend deux chaînes de polypeptides qui, sous forme glycosylée, présentent une masse moléculaire apparente d'environ 16 kD et 18 kD;
(d) comprend deux chaînes de polypeptides qui, sous forme non glycosylée, présentent une masse molécu-

laire apparente d'environ 14 kD à 16 kD.

4. Procédé selon l'une quelconque des revendications précédentes, dans lequel la cellule-hôte est une cellule-hôte d'E. coli.

5. Procédé pour produire une dispositif ostéogène, comprenant l'étape qui consiste à disposer la protéine selon l'une quelconque des revendications précédentes dans une matrice.

6. Procédé selon la revendication 5, dans lequel le dispositif est destiné à être implanté chez un mammifère et comprend:

(a) une matrice biocompatible et biodégradable in vivo qui définit des pores d'une dimension suffisante pour permettre l'influx, la prolifération et la différenciation de cellules progénitrices migrant depuis le corps du dit mammifère;
(b) la protéine disposée dans la dite matrice et accessible aux dites cellules.

7. Procédé selon la revendication 6, dans lequel ladite matrice comprend un matériau en particules étroitement empilées dont la taille est comprise dans la plage de 70 à 420 µm.

8. Procédé selon l'une quelconque des revendications 5 à 7, dans lequel ladite matrice comprend:

(a) de l'os allogène, par exemple de l'os allogène déminéralisé, débarrassé de ses protéines, en particules;
(b) de l'os xénogène déminéralisé, débarrassé de ses protéines, en particules, déglycosylé (par exemple traité avec une protéase);
(c) de l'os xénogène déminéralisé, débarrassé de ses protéines, en particules, traité par HF;
(d) des matériaux sélectionnés parmi le collagène, l'hydroxyapatite, les phosphates de calcium (par exemple le triphosphate de calcium) et des polymères comprenant des monomères d'acide glycolique et/ou d'acide lactique;
(e) un matériau solide, conservant sa forme, en particules adhérant lâchement les unes aux autres, par exemple le collagène;
(f) un solide poreux;
(g) un tissu mastiqué, par exemple du muscle, ou
(h) la cavité médullaire d'os allogène.

9. Procédé selon l'une quelconque des revendications 5 à 7, dans lequel le dispositif est destiné à être utilisé en thérapie, par exemple pour induire la formation locale de cartilage et/ou d'os endochondrien ou hétérotope chez un mammifère, par implantation du dispositif chez un mammifère, en un site accessible à des cellules progénitrices migrantes pour le traitement périodontal, la réparation de cartilage, l'accélération de réparations après greffes allogènes et pour le traitement de l'ostéoarthrite ou pour la réparation de défauts d'os porteurs, la correction de fractures sans union ou d'anomalies squelettiques, dentaires ou crano-faciales acquises ou congénitales.

10. Procédé selon la revendication 9, dans lequel le dispositif est destiné à être utilisé pour induire de l'os hétérotope façonné, la forme de l'os hétérotope se conformant à celle du dispositif implanté.

FIG. 1A-1

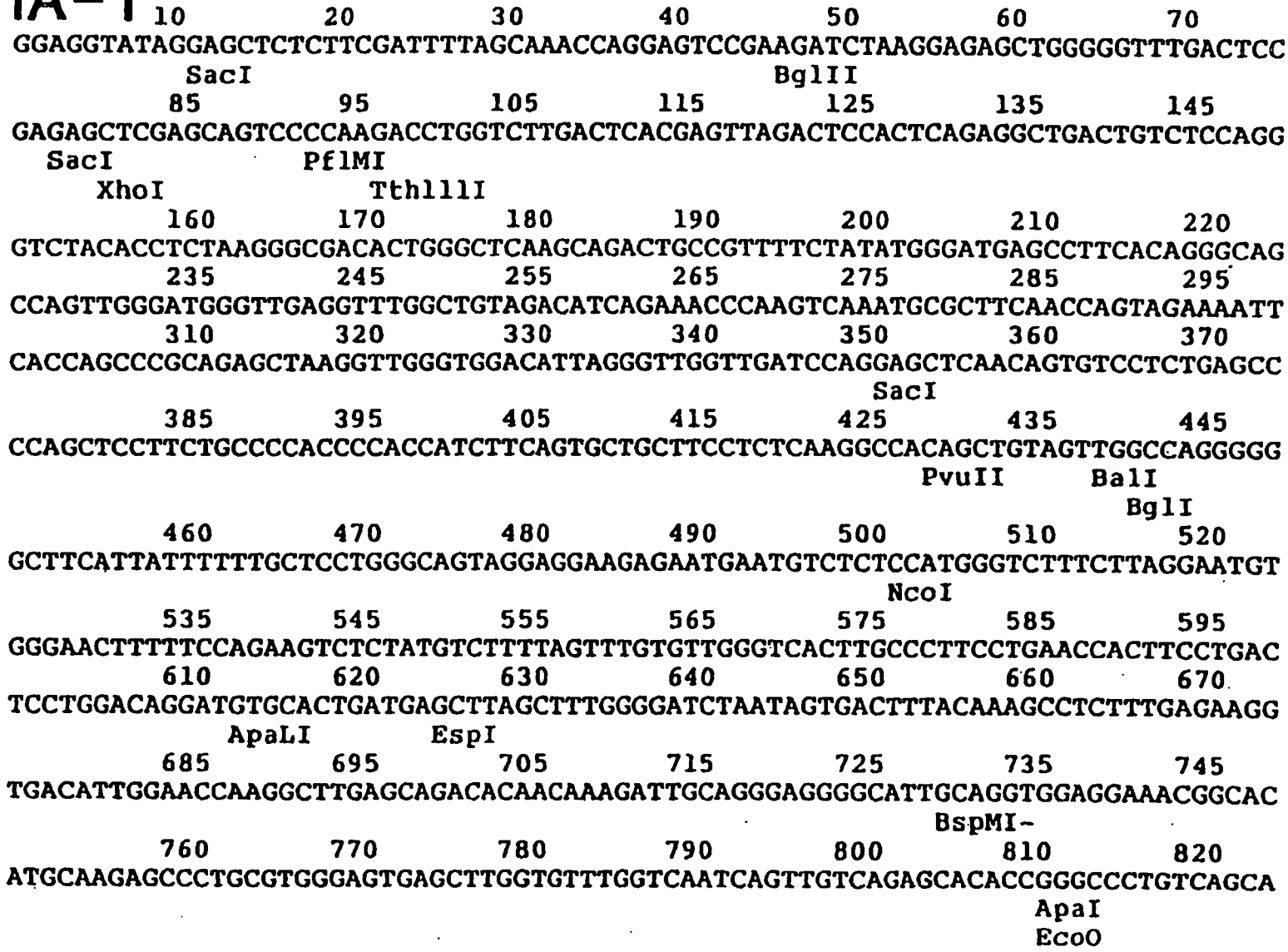


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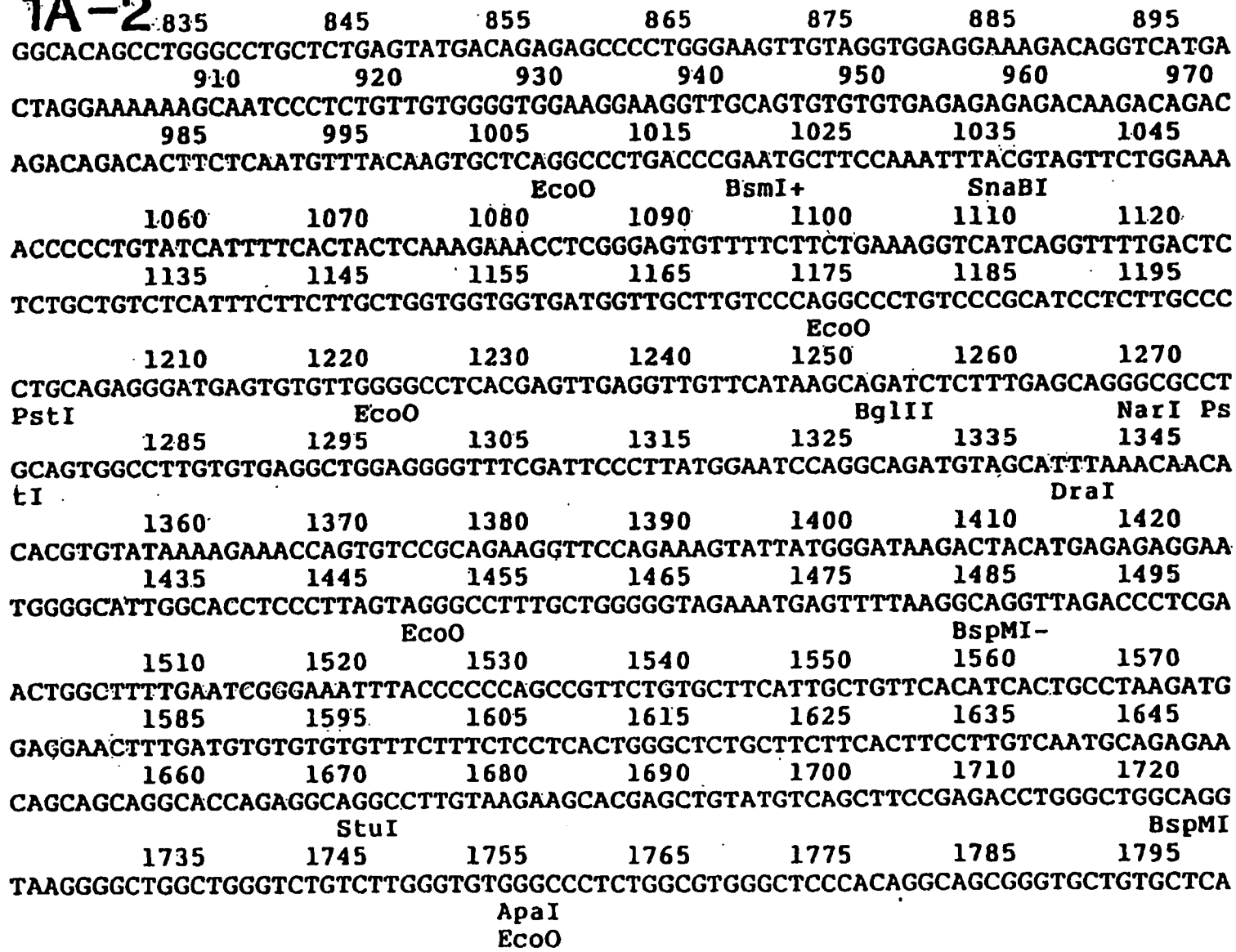
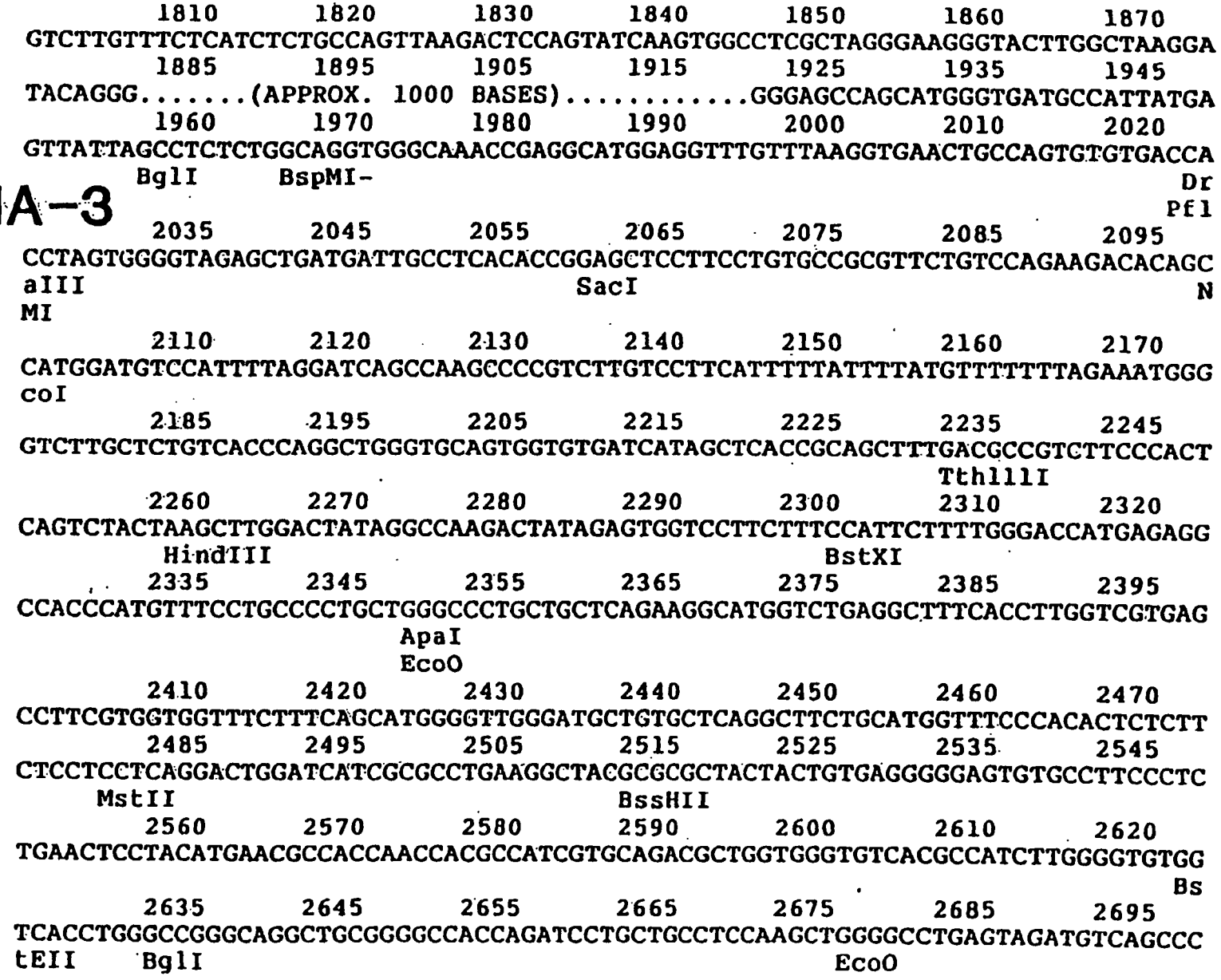
FIG. 1A-2



EP 0 714 665 B1

FIG. 1A–3



EP 0 714 665 B1

FIG. 1A-4

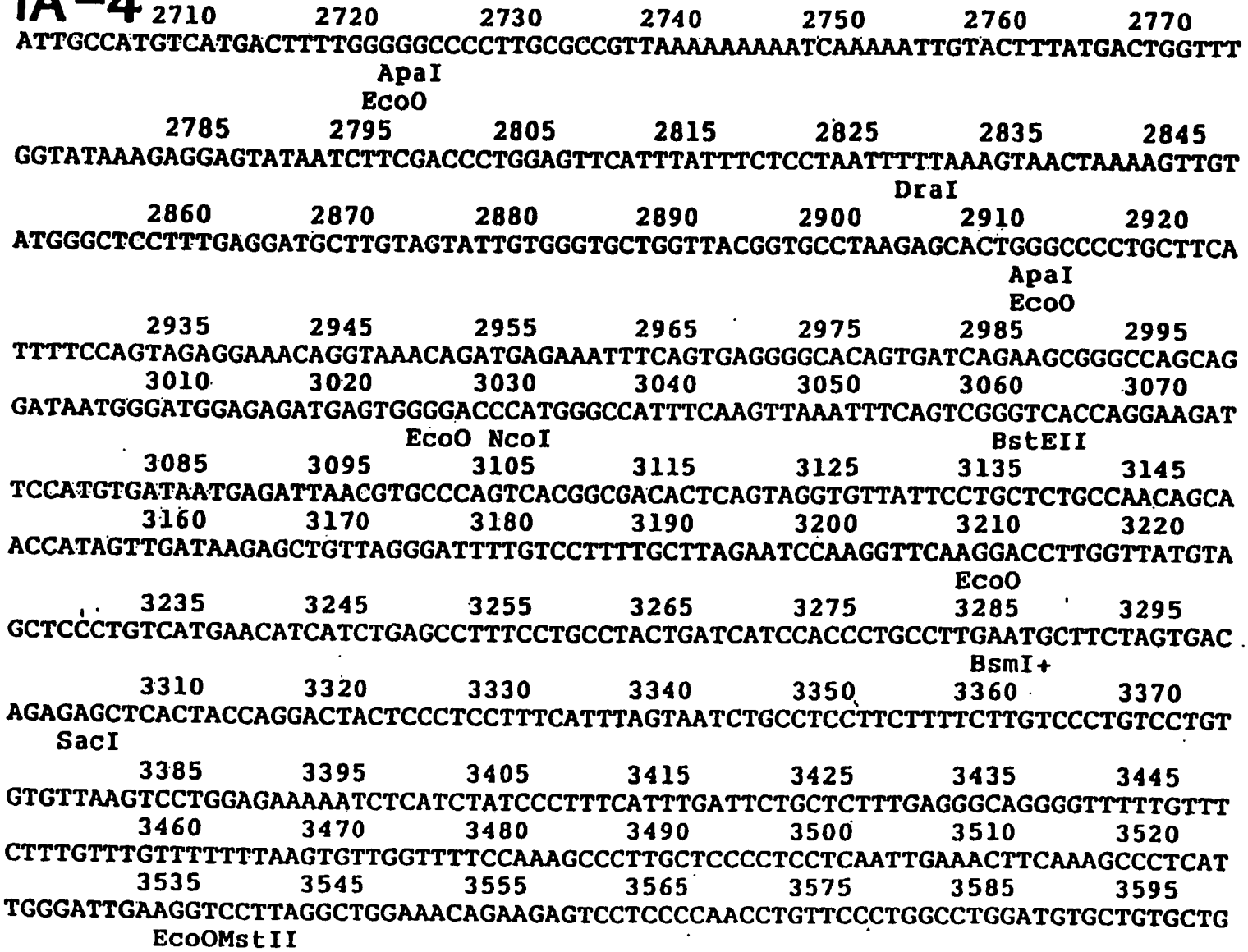


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FIG. 1A-5

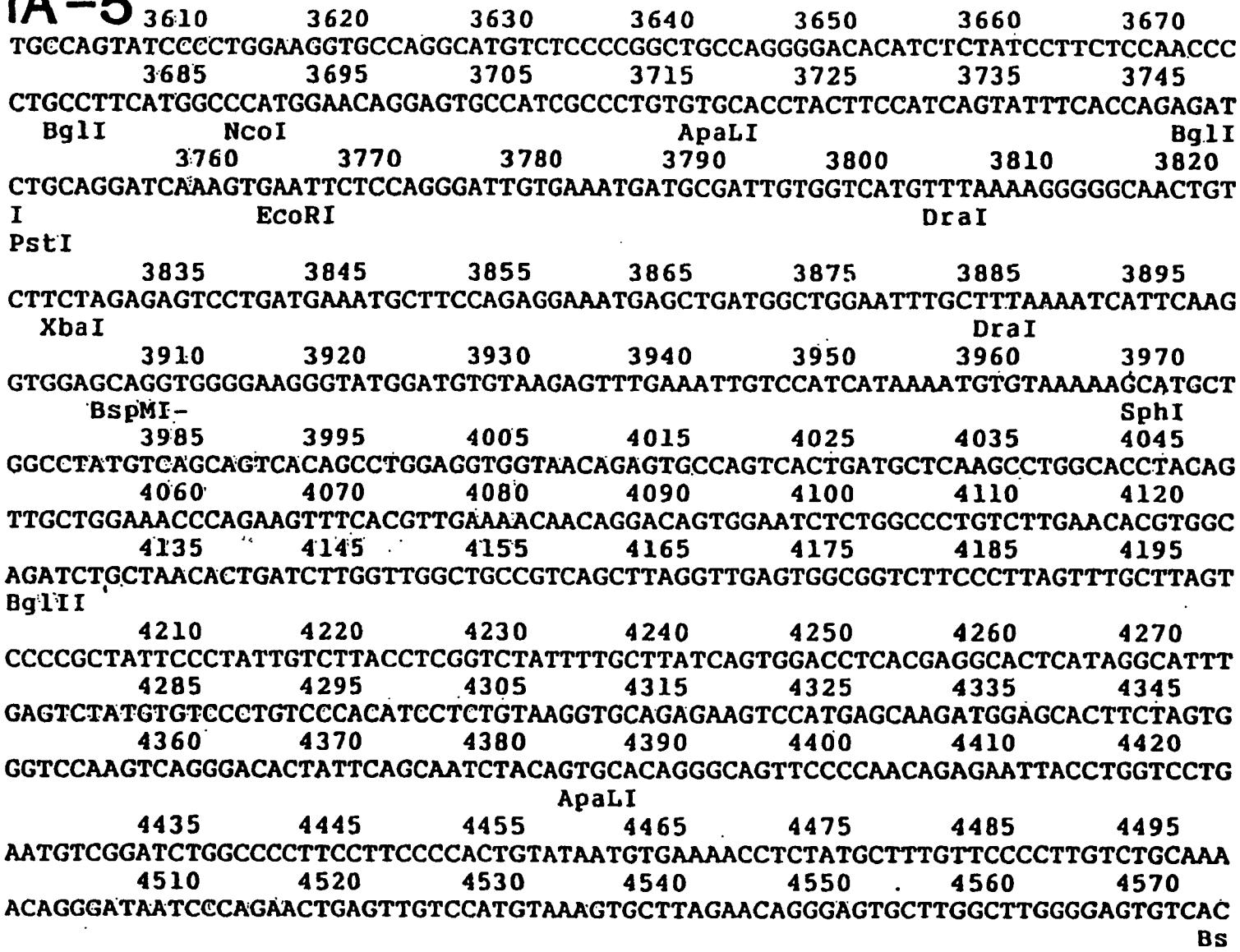

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FIG. 1A-6

```
        4585       4595       4605       4615       4625       4635       4645
CTGCAGTCATTCATTATGCCCAGACAGGATGTTTCTTTATAGAAACGTGGAGGCCAGTTAGAACGACTCACCGCT
pMI+
PstI
        4660       4670       4680       4690       4700       4710       4720
TCTCACCACTGCCCATGTTTTGGTGTGTGTTTCAGGTCCACTTCATCAACCCGGAAACGGTGCCCAAGCCCTGCT
             PflMI
        4735       4745       4755       4765       4775       4785       4795
GTGCGCCCACGCAGCTCAATGCCATCTCCGTCCTCTACTTCGATGACAGCTCCAACGTCATCCTGAAGAAATACA
        4810       4820       4830       4840
GAAACATGGTGGTCCGGGCCTGTGGCTGCCACTAGCTCCTCCGA
```

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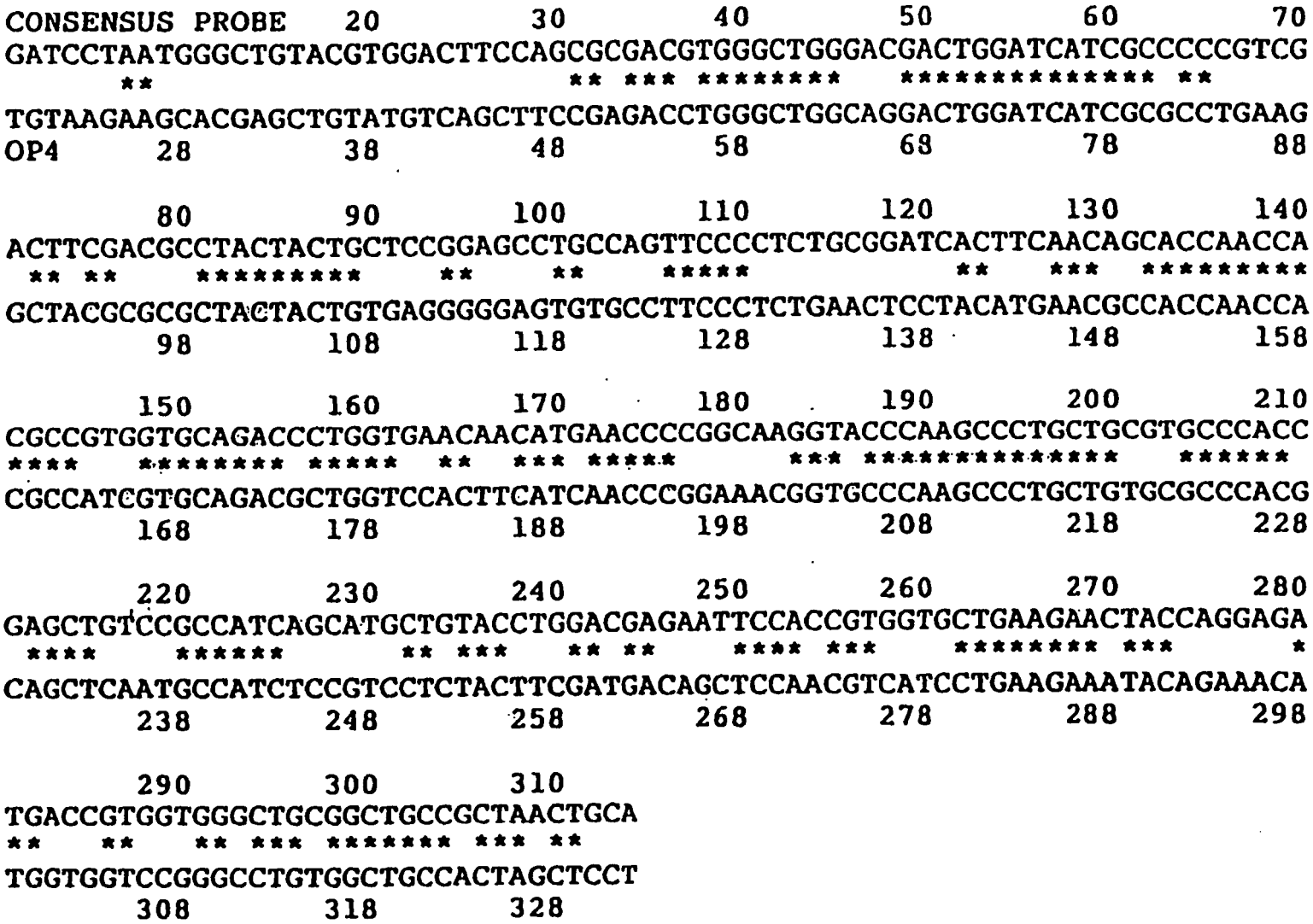
FIG. 1B



EP 0 714 665 B1

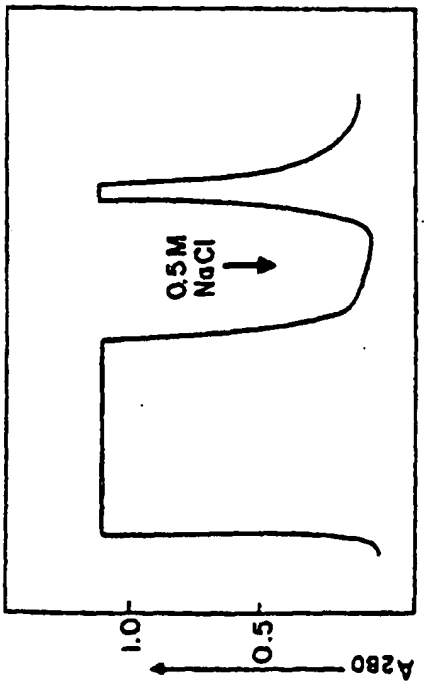


FIG. 2A

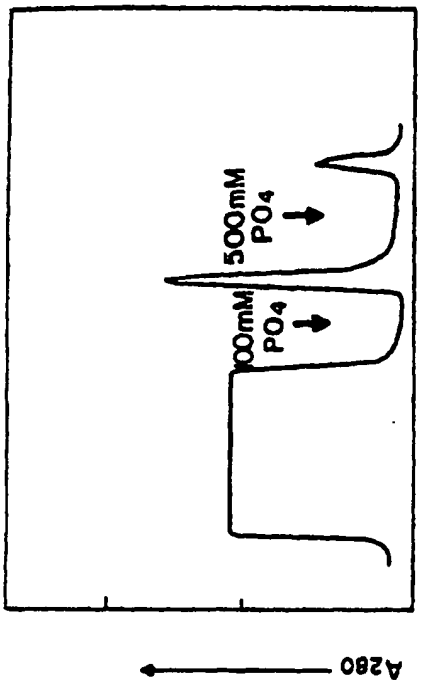


FIG. 2B

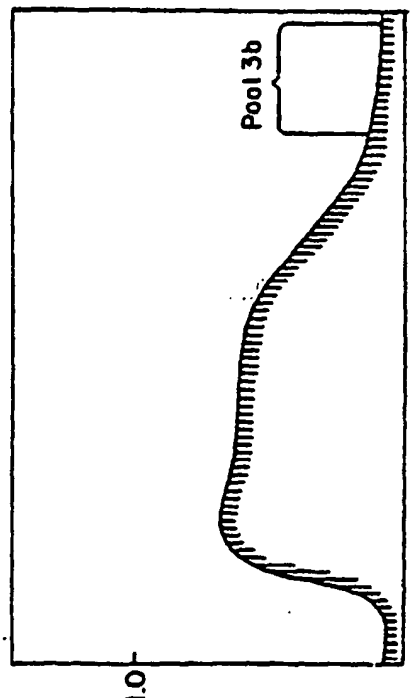


FIG. 2C

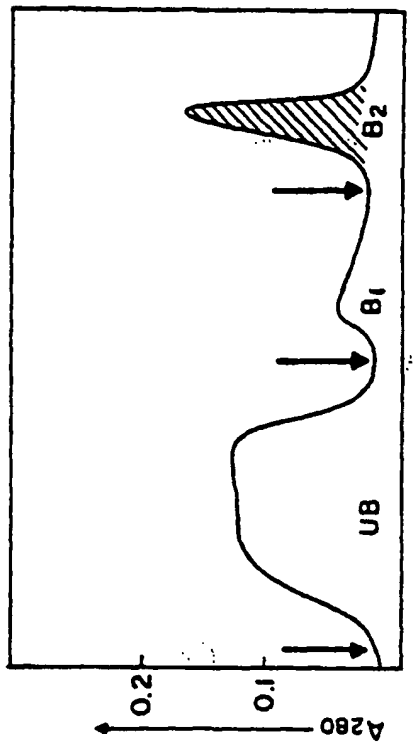


FIG. 2D

FIG. 3A FIG. 3B

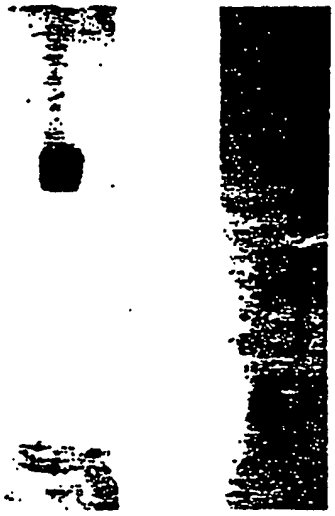


FIG. 4A FIG. 4B

FIG. 5A

FIG. 5B

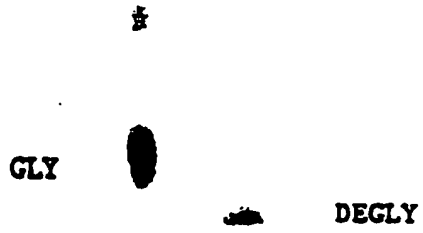
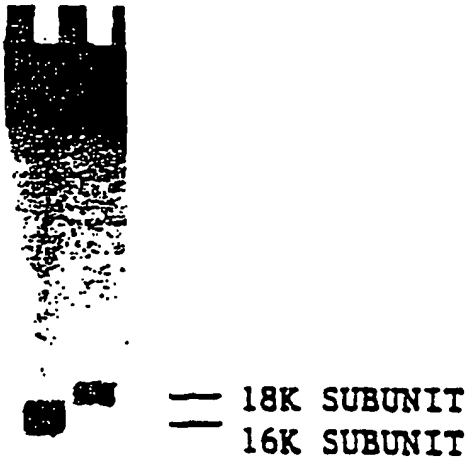


FIG. 6 A FIG. 6 B FIG. 6 C FIG. 6 D FIG. 6 E

FIG. 15



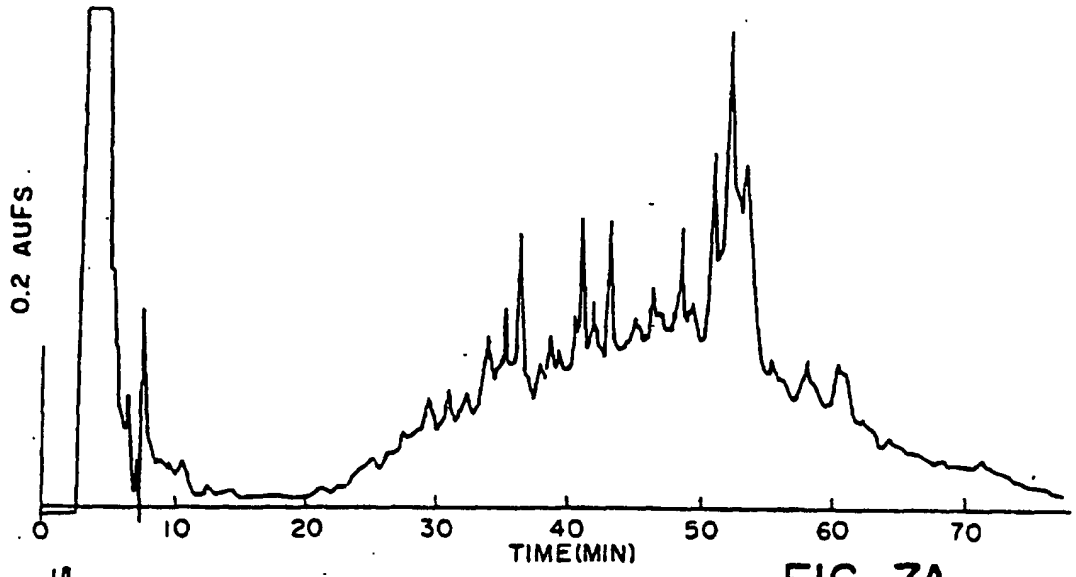


FIG. 7A

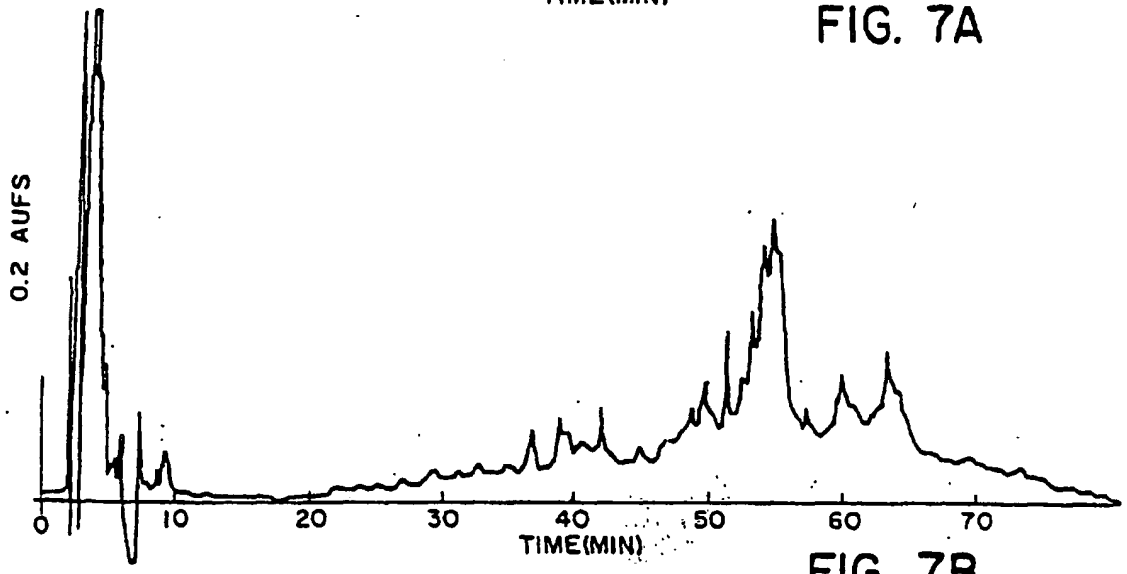


FIG. 7B

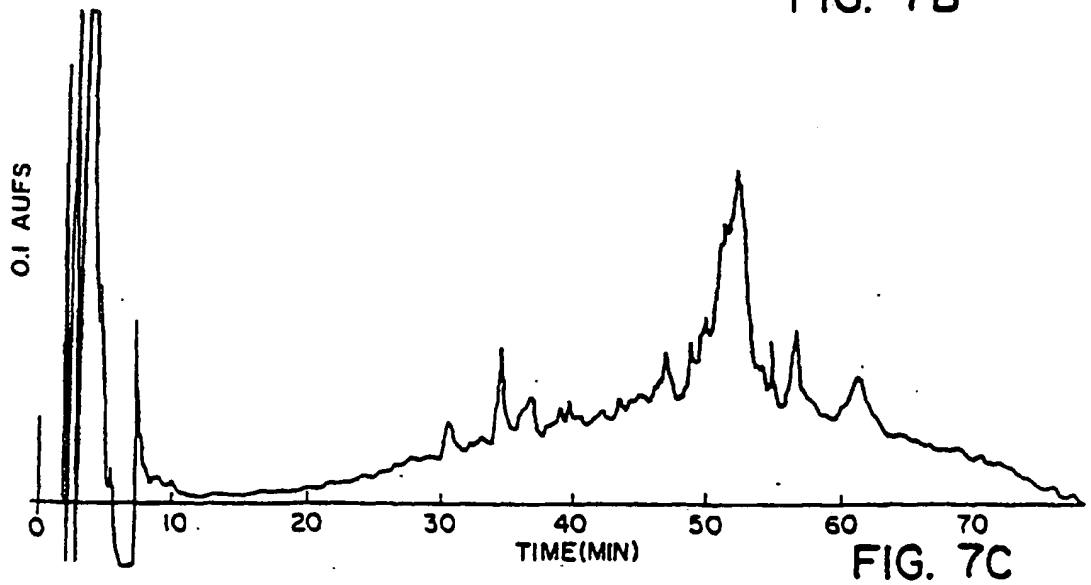


FIG. 7C

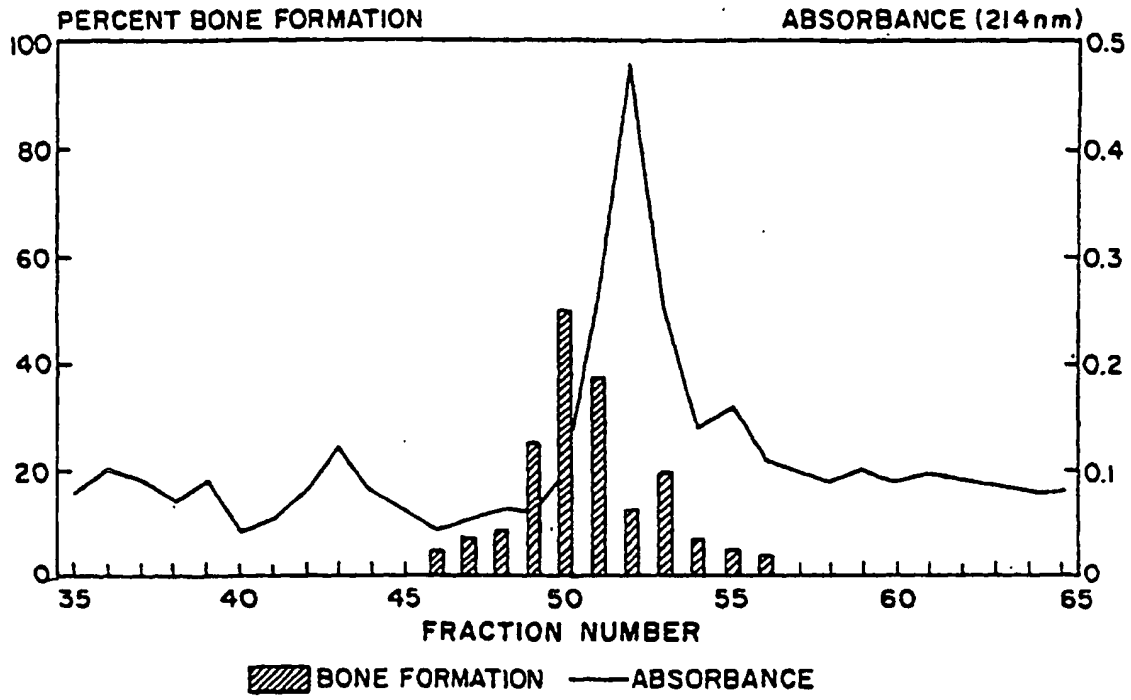

PERCENT BONE FORMATION
ABSORBANCE (214nm)
100
80
60
40
20
0
0.5
0.4
0.3
0.2
0.1
0
35
40
45
50
55
60
65
FRACTION NUMBER
BONE FORMATION
ABSORBANCE

FIG. 8

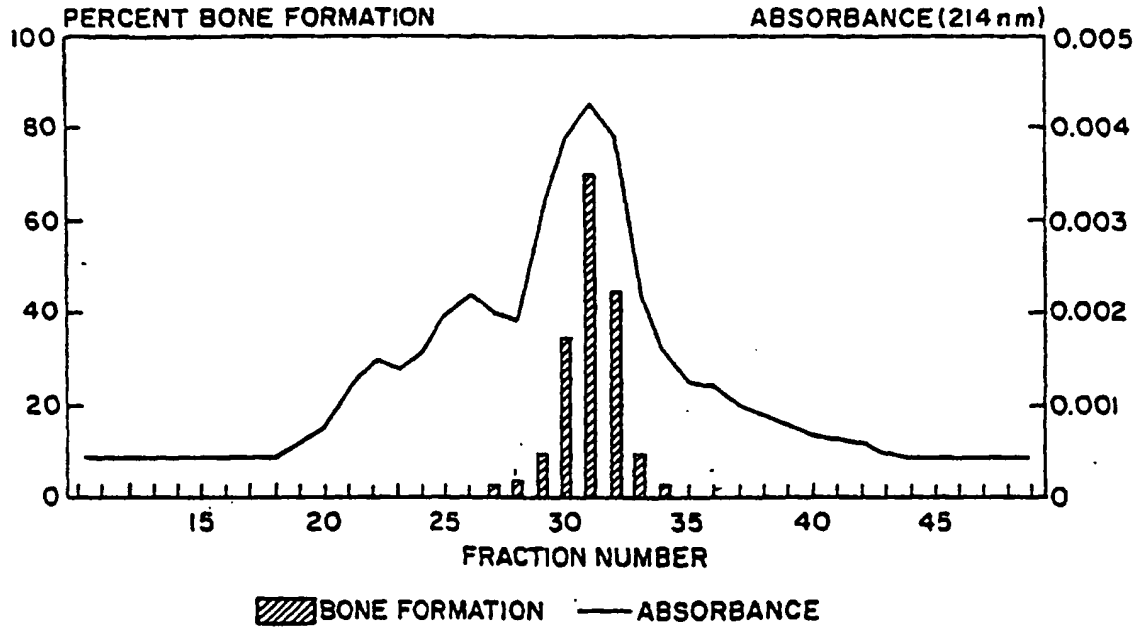

PERCENT BONE FORMATION
ABSORBANCE (214nm)
100
80
60
40
20
0
0.005
0.004
0.003
0.002
0.001
0
15
20
25
30
35
40
45
FRACTION NUMBER
BONE FORMATION
ABSORBANCE

FIG. 9

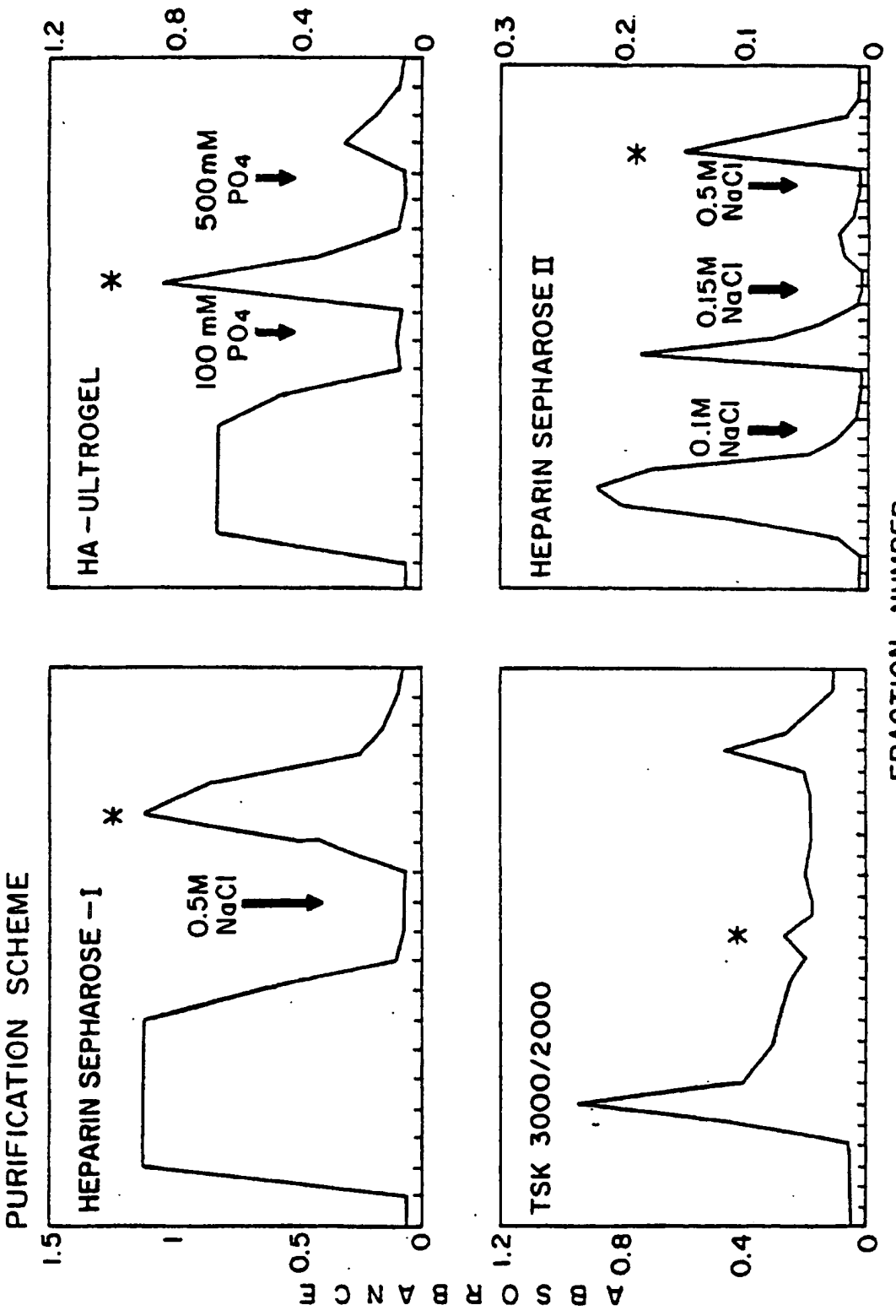

PURIFICATION SCHEME
HEPARIN SEPHAROSE -I
0.5M NaCl
HA -ULTROGEL
100 mM PO4
500mM PO4
TSK 3000/2000
HEPARIN SEPHAROSE II
0.1M NaCl
0.15M NaCl
0.5M NaCl
ABSORBANCE
FRACTION NUMBER

FIG. 10

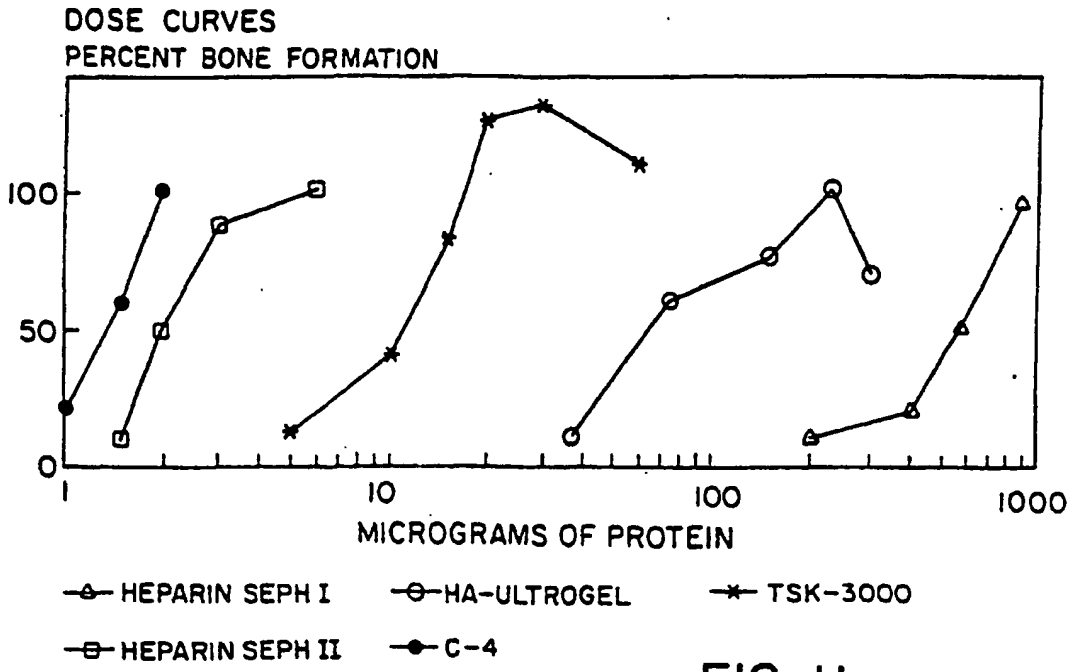

DOSE CURVES
PERCENT BONE FORMATION
100
50
0
1
10
100
1000
MICROGRAMS OF PROTEIN
HEPARIN SEPH I
HA-ULTROGEL
TSK-3000
HEPARIN SEPH II
C-4

FIG. 11

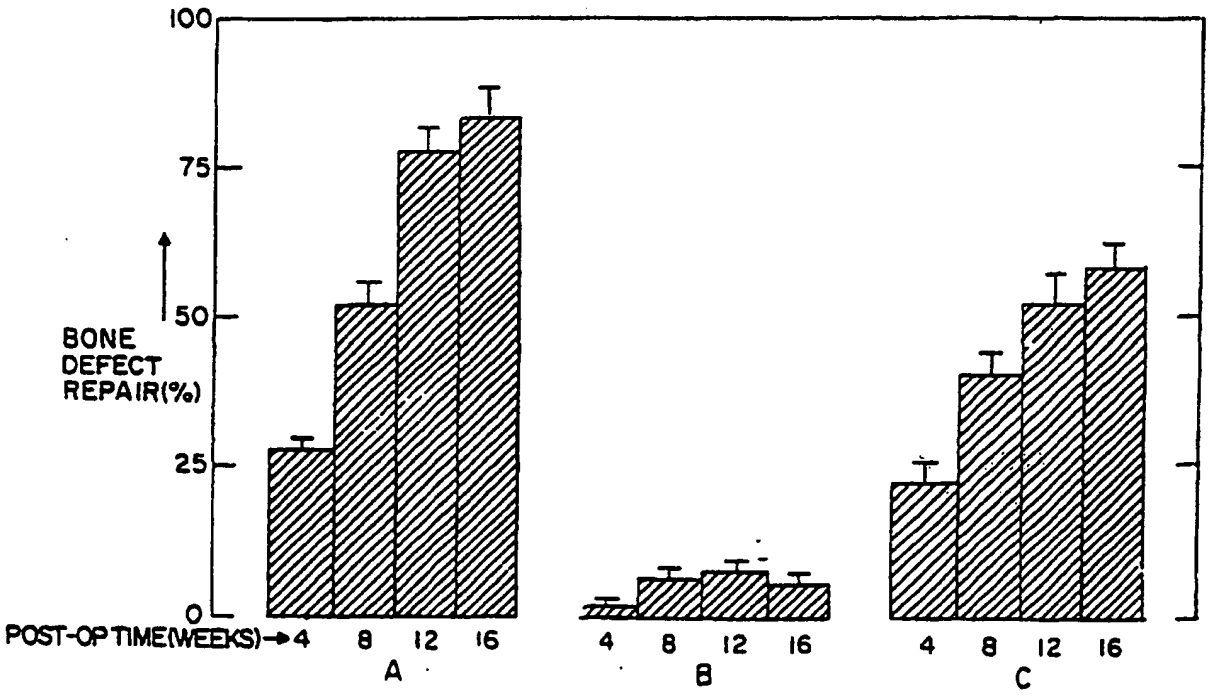
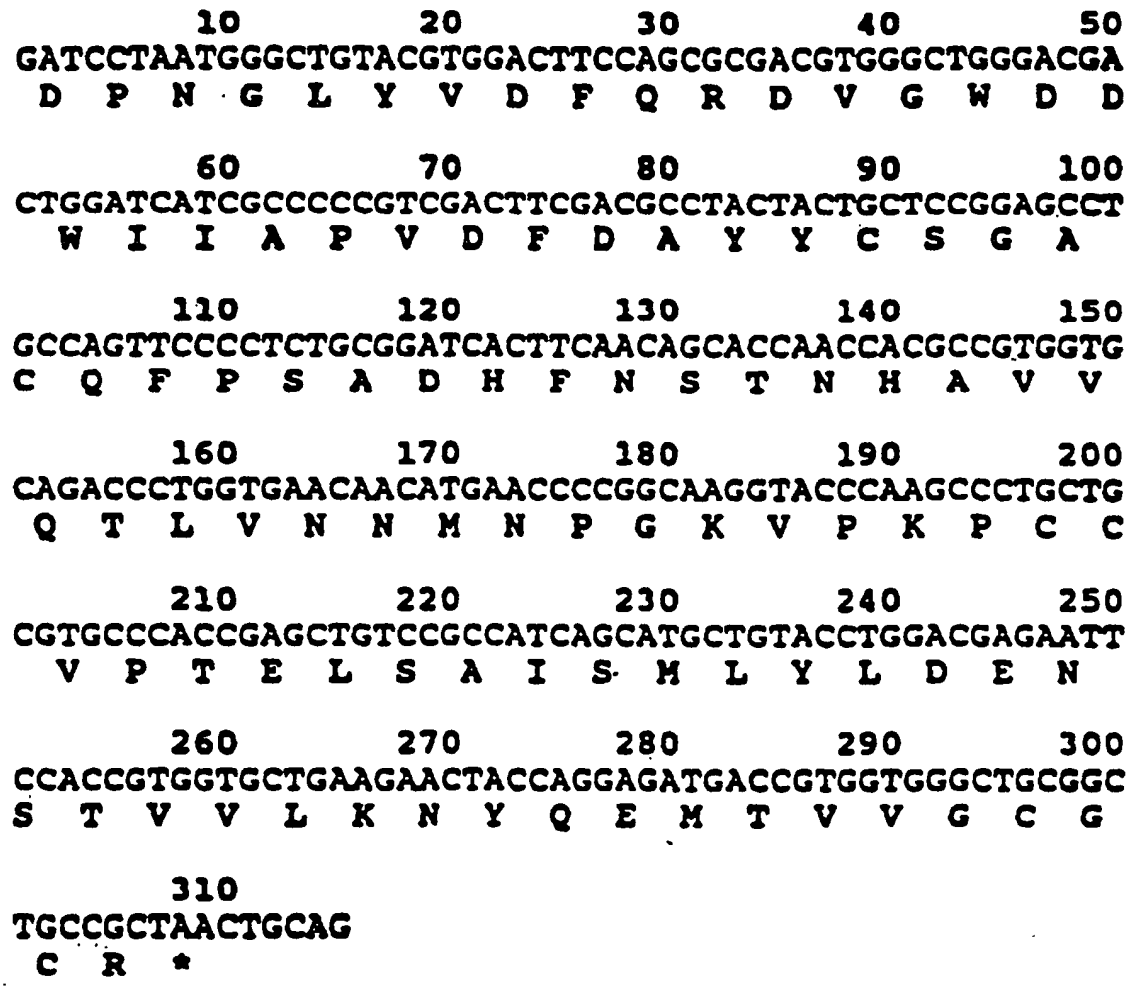

100
75
50
25
0
BONE
DEFECT
REPAIR(%)
POST-OP TIME(WEEKS)→4
8
12
16
A
4
8
12
16
B
4
8
12
16
C

FIG. 12

FIG. 13


10 20 30 40 50
GATCCTAATGGGCTGTACGTGGACTTCCAGCGCGACGTGGGCTGGGACGA
D P N G L Y V D F Q R D V G W D D
60 70 80 90 100
CTGGATCATCGCCCCCGTCGACTTCGACGCCTACTACTGCTCCGGAGCCT
W I I A P V D F D A Y Y C S G A
110 120 130 140 150
GCCAGTTCCCCTCTGCGGATCACTTCAACAGCACCAACCACGCCGTGGTG
C Q F P S A D H F N S T N H A V V
160 170 180 190 200
CAGACCCTGGTGAACAACATGAACCCCGGCAAGGTACCCAAGCCCTGCTG
Q T L V N N M N P G K V P K P C C
210 220 230 240 250
CGTGCCCACCGAGCTGTCCGCCATCAGCATGCTGTACCTGGACGAGAATT
V P T E L S A I S M L Y L D E N
260 270 280 290 300
CCACCGTGGTGCTGAAGAACTACCAGGAGATGACCGTGGTGGGCTGCGGC
S T V V L K N Y Q E M T V V G C G
310
TGCCGCTAACTGCAG
C R *

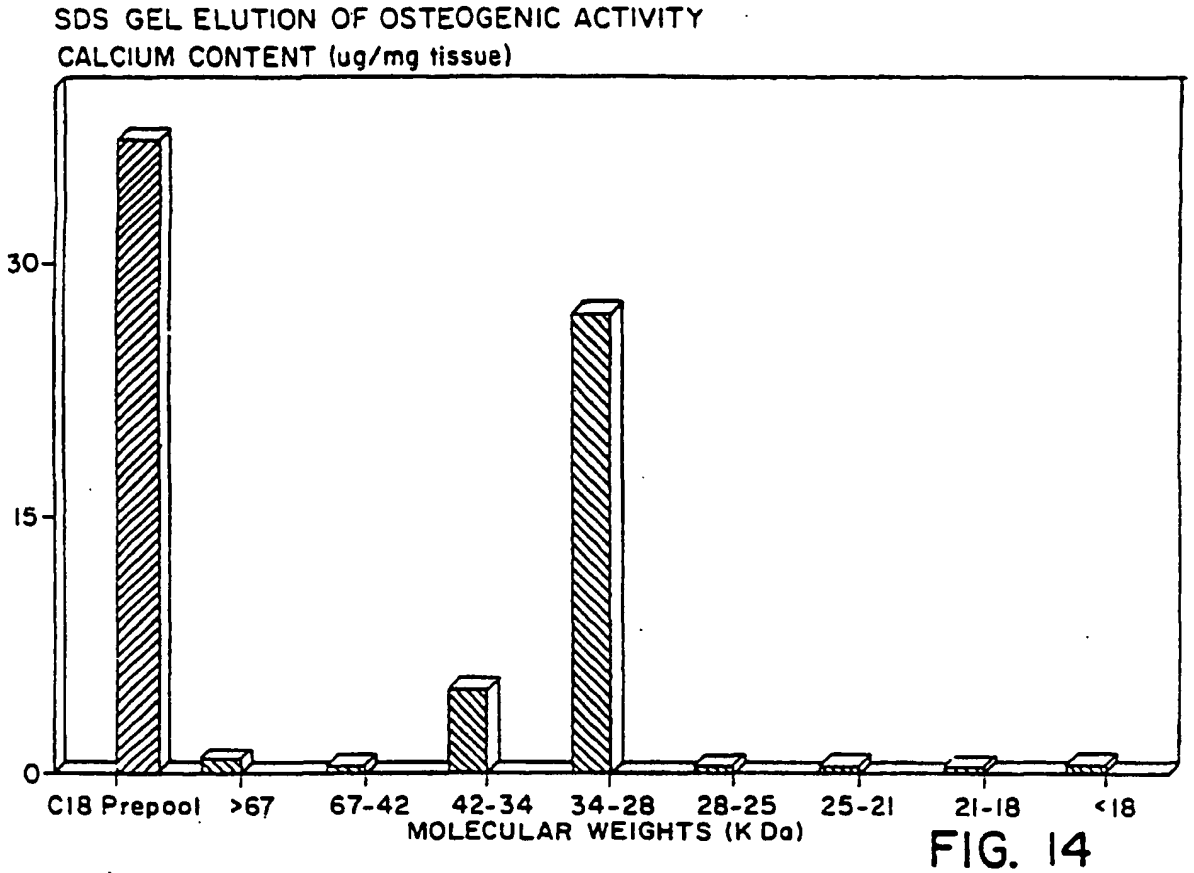

SDS GEL ELUTION OF OSTEOGENIC ACTIVITY
CALCIUM CONTENT (ug/mg tissue)
30
15
0
C18 Prepool
>67
67-42
42-34
34-28
28-25
25-21
21-18
<18
MOLECULAR WEIGHTS (K Da)

FIG. 14

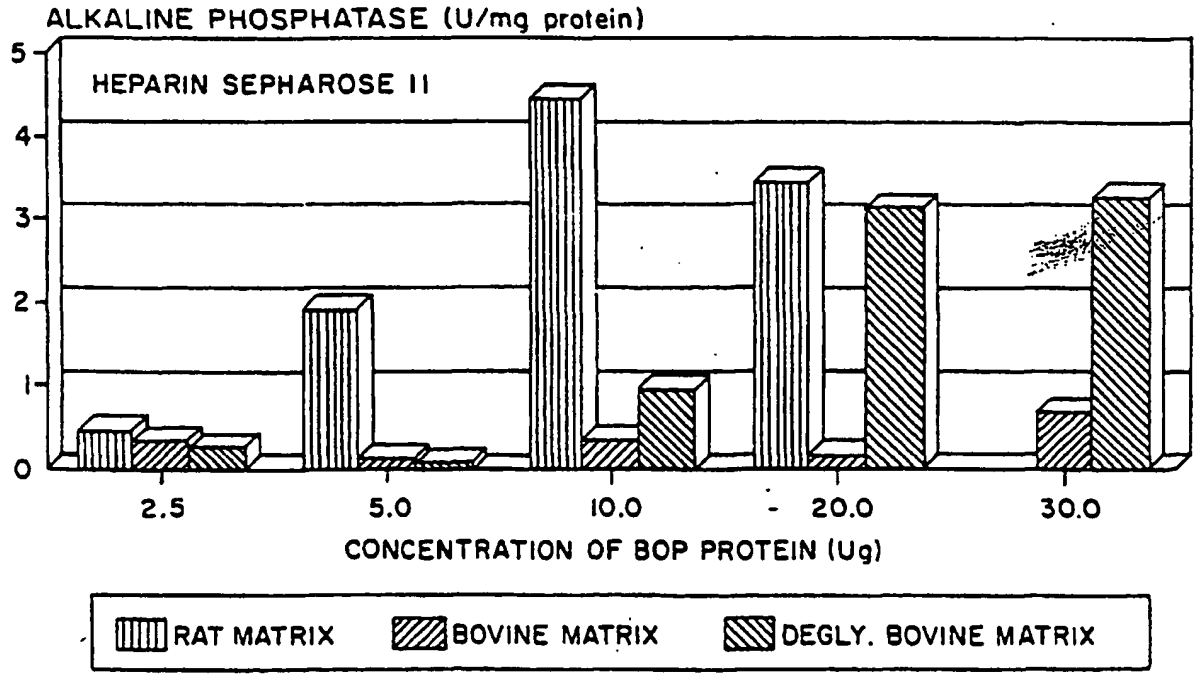

ALKALINE PHOSPHATASE (U/mg protein)
HEPARIN SEPHAROSE II
5
4
3
2
1
0
2.5
5.0
10.0
20.0
30.0
CONCENTRATION OF BOP PROTEIN (Ug)
RAT MATRIX
BOVINE MATRIX
DEGLY. BOVINE MATRIX

FIG. 18

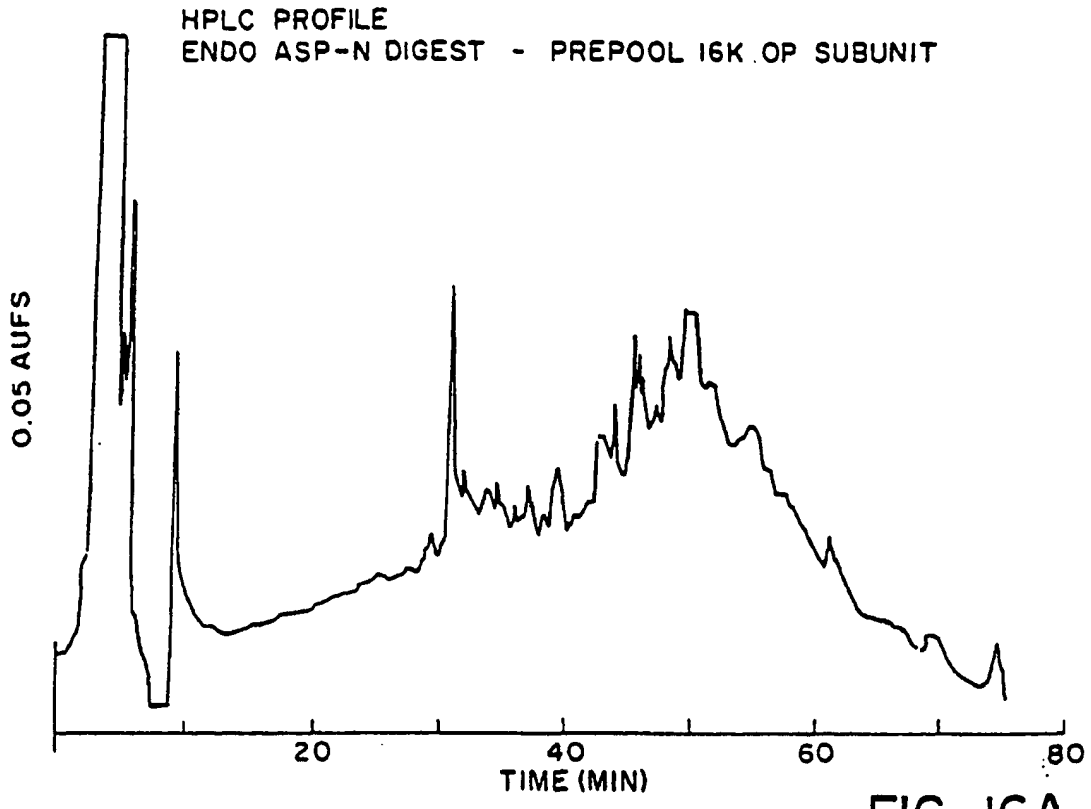


FIG. 16A

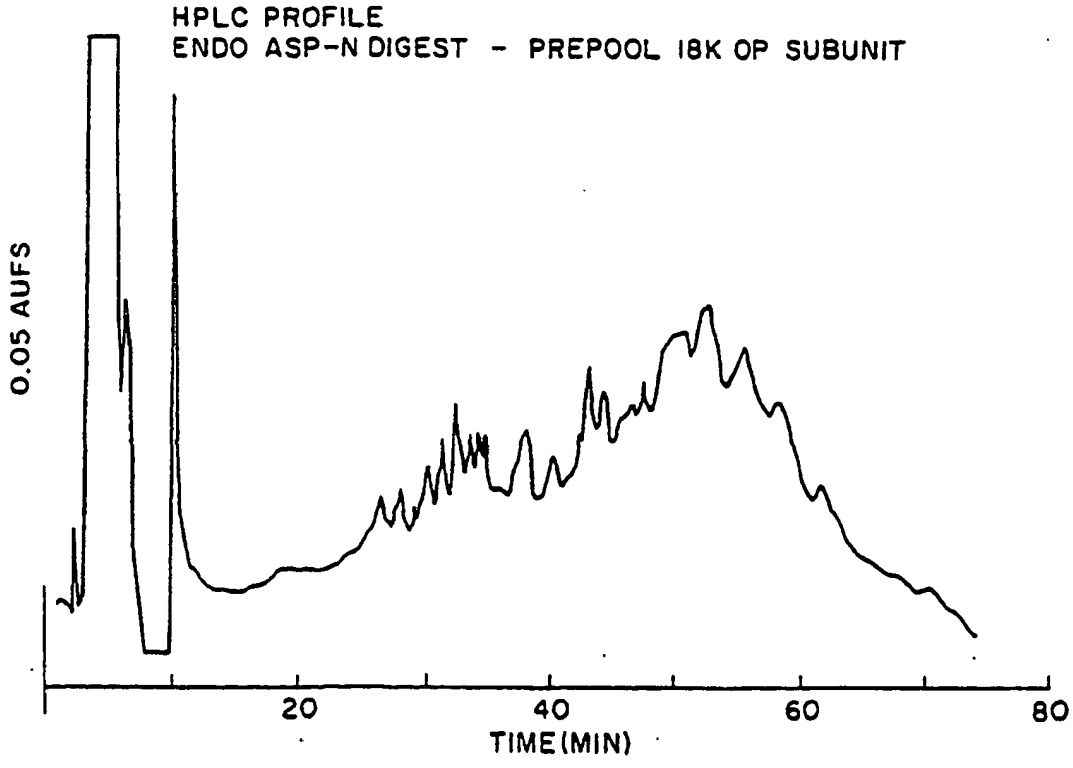


FIG. 16B

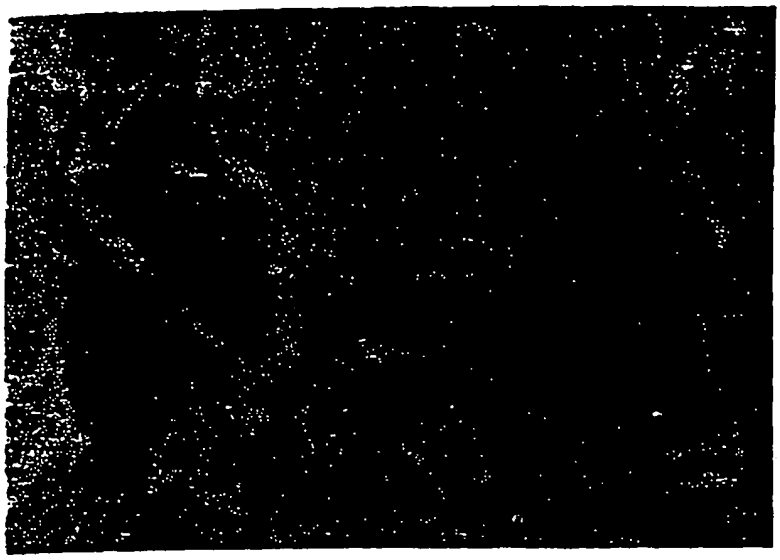


FIG. 17A

FIG. 17B

FIG. 17C

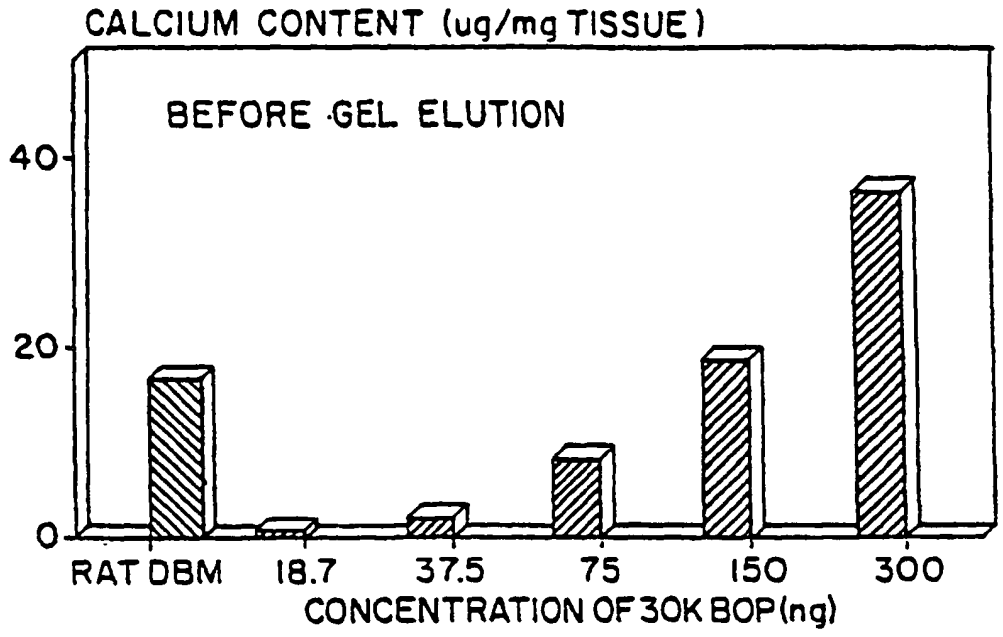


FIG. 19A

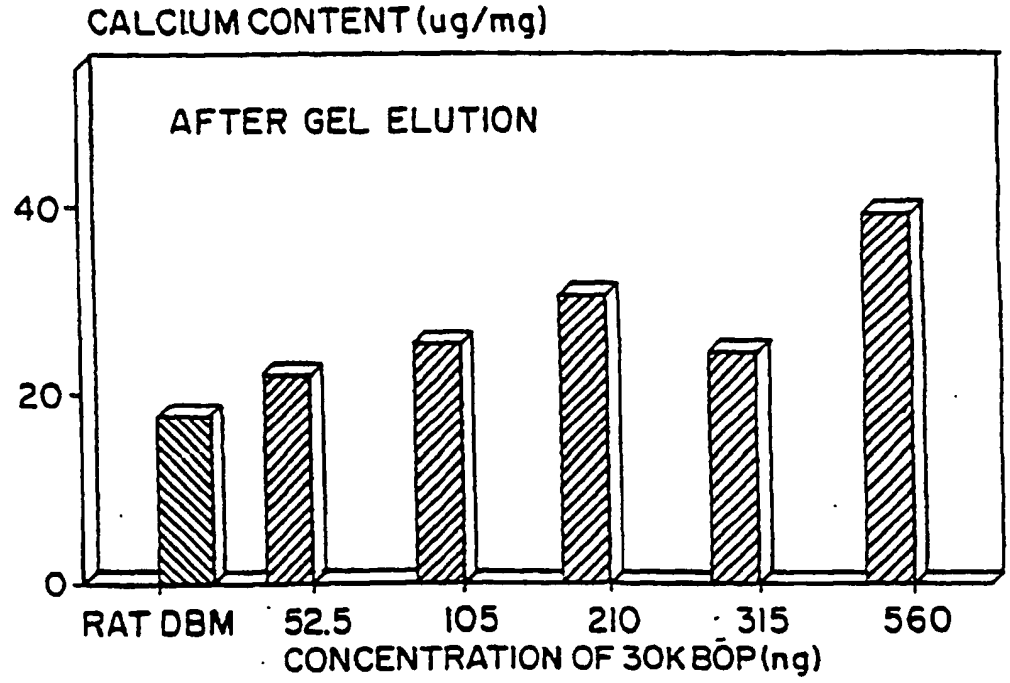


FIG. 19B